BIOFILM FORMATION IN ESCHERICHIA COLI AND REGULATORY GENE EXPRESSION VIA QUORUM SENSING SYSTEMS

by

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ABSTRACT

Bacterial biofilms are microbial communities that adhere to abiotic or biotic surfaces. Biofilm formation (BF) studies in *Escherichia coli* have primarily concentrated on uropathogenic *E. coli*, commensal K-12 and enterohemorrhagic *E. coli* O157:H7. This does not include the vast diversity of environmental strains.

Quorum sensing (QS) is a means by which bacteria can communication with one another through the production of signalling molecules. The autoinducer 2 (AI-2) QS system is utilized by *E. coli* and several other bacterial species for controlling gene expression. The role of AI-2 in *E. coli* BF varies among different strains. For example in the K-12 strain, AI-2 regulates motility, and thus can affect BF; whereas in O157:H7, AI-2 has a more metabolic role. Interestingly, in strain O157:H7, motility is controlled by a newly discovered QS system regulated by the autoinducer 3 (AI-3) molecule plus the mammalian hormones epinephrine (Epi) and norepinephrine (Ne).

The purpose of this study was to investigate the ability of a panel of environmental E. coli strains to form biofilms and to determine whether QS is involved in the process. A new pathotype of *E. coli*, adherent invasive *E. coli* (AIEC) which is associated with Crohn's disease was included in the investigation. Study 1 sought to determine whether BF under different media conditions correlated with the presence of genes involved in the AI-2 QS system or adhesin factors. Media conditions were the principal variable affecting the BF. Study 2 examined the role of the AI-2 and AI-3/Epi/Ne QS systems in

motility and BF by the AIEC strain. It was discovered that the AI-3 system is involved in motility; whereas the AI-2 system had no effect on BF or motility. In Study 3, microarray gene expression analysis and invasion assays were performed using *qseB* or *qseC* mutants. These genes encode the two-component regulatory system recognizing AI-3 or its cognate, epinephrine. Our findings indicate that alternative pathways likely account for the BF observed for the *qseB* and *qseC* mutants. It was concluded that the AI-3/Epi/Ne QS system partially controls AIEC motility and the invasion of epithelial cells.

DEDICATION

This thesis is dedicated to my parents, Gladys Doria (may she rest in peace) and Bartolo Hernandez. Due to their dedication and support I have been able to accomplish all the things in my live, and thanks to them I have become the person that stands today.

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FOREWORD

This dissertation is written in manuscript style and is composed of three manuscripts. All the manuscripts are ready for publication in journals related to gut microbiology.

The authors of the manuscripts are:

Manuscript I: J. D. Hernandez-Doria, T. R. de Kievit, D. O. Krause

Manuscript II: J. D. Hernandez-Doria, T. R. de Kievit, D. O. Krause

Manuscript III: J. D. Hernandez-Doria, T. R. de Kievit ,D.O. Krause

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LIST OF ABBREVIATIONS

AdrA	AgfD-regulated protein
AE	Attaching and effacing
Afa	Afimbrial adhesins
Ag43	Antigen 43 protein
AHL	Acyl homoserine lactone
AI-2	Autoinducer 2
AI-3	Autoinducer 3
Aida	Adhesin involved in diffuse adherence
AIEC	Adherent and invasive *Escherichia coli*
ANOVA	Analysis of variance
APEC	Avian pathogenic *E. coli*
ATG16L1	Autophagy related 16 like 1
BcsA	Cellulose synthase catalytic subunit A
BcsB	Cellulose biosynthesis protein B

Bma	M afimbrial adhesin
CD	Crohn’s disease
C-di-GMP	Cyclic diguanosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CEACAM6	Carcinoembryonic antigen related cell adhesion molecule 6
CFU	Colony forming unit
CRF	Clarified rumen fluid
CRH	Corticotrophin releasing hormone
CV	Crystal violet
DAVID	Database for annotation, visualization and integrate discovery
DNA	Deoxyribonucleic acid
DPD	4,5-dihydroxy-2,3-pentanedione
EHEC	Enterohemorrhagic *Escherichia coli*
EnvZ	Envelope protein Z
EPEC	Enterophatogenic *E. coli*
Epi	Epinephrine

FAM	5-carboxyfluerescein
FC	Fold Change
FimA	Major type 1 subunit fimbrim
FimB	Regulator for FimA
FimC	Periplasmic chaperone, required for type 1 fimbriae
FimD	Major type 1 subunit fimbrin
FimF	Fimbrial morphology
FimH	Minor fimbrial subunit, D-mannose specific adhesin H
FlhDC	Master flagella regulon
FliA	RNA polymerase sigma factor 28
FliC	Filament structural protein, flagellin C
H_2O_2	Hydrogen peroxide
HAI-1	Homoserine autoinducer 1
HUS	Haemolytic uremic syndrome
IBD	Inflammatory bowel disease
IL	Interleukins

IFN-γ	Interferon-gamma
LacZ	β-galactosidase monomer
LB	Luria bertani
Lee	Locus of enterocyte and effacement
Lsr	LuxS regulated genes
LsrK	LuxS-regulated kinase
LuxI	Luciferase protein 1
LuxN	Luciferase protein N
LuxP	Luciferase protein P
LuxQ	Luciferase protein Q
LuxS	Luciferase gene S
LuxU	Luciferase protein U
M9	Minimal medium 9
MLST	Multi locus sequence typing
MqsR	Motility quorum sensing regulator gene
NaCL	Sodium Chloride

Ne	Norepinephrine
NFQ	Non fluorescent quencher
NF-kB	Nuclear factor kappa
NOD2	Nucleotide-binding oligomerization domain containing 2
OD	Optical density
OmpC	Outer membrane protein C
OmpF	Outer membrane protein F
OmpR	Outer membrane protein R
Pap	Pyelonephritis associated pili
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Pfs	5’methylthioadenosine/s-adenosylhomocysteine nucleosidase monomer
PVC	Polyvinyl Chloride
PWF	Pig water feces
QS	Quorum sensing
QseB	Quorum sensing *E. coli* B, response regulator

QseC	Quorum sensing *E. coli* C, sensor histidine kinase
RMA	Robust multiarray average algorithm
RNA	Ribonucleic acid
RpoA	RNA polymerase alpha subunit
RT-PCR	Reverse transcription of polymerase chain reaction
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SAS	Statistical analysis system
Sfa	Sialic acid fimbrial adhesin
STEC	Shigatoxin producing strains
S-THMF	(2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran
TNF-α	Tumor necrosis factor-alpha
UPEC	Uropathogenic Escherichia coli
UTI	Urinary tract infections

GENERAL INTRODUCTION

The vast majority of bacteria live in a consortia of microorganisms instead of being free living unicellular entities. This state is usually referred to as the biofilm lifestyle, and was introduced more than two decades ago (Costerton et al., 1987). These sessile structures can be composed of single bacterial species or multiple species, and may also contain eukaryotes cells (Costerton et al., 1987). In the biofilm state, bacteria can actually survive in diverse environments and be protected against dangerous conditions such as targeting by white blood cells, bacteriophages, and antibiotics, (Costerton et al., 1987). Biofilms are related to persistent diseases, including periodontitis, pneumonia and nosocomial infections like urinary tract infections (Costerton et al., 1999). A growing concern is the fact that bacterial biofilms are resistant to antimicrobial treatments and host immune defences (Costerton et al., 1999). For instance, it is known that the Gram negative bacteria *Pseudomonas aeruginosa* is often related with cystic fibrosis lung infections, and antibiotic treatments against it are ineffective so the rate of infection recurrence is high (Wagner and Iglewski, 2006). *P. aeruginosa* growing as a biofilm survives higher antibiotic concentrations than its planktonic counterpart, because of peptide glucans which are part of the biofilm matrix, thus evading full penetration of the antibiotics (Hassett et al., 2010). Today this bacterium has become one of the best studied biofilm models in the field.

Dental biofilm contain more than 500 bacterial species, many of which have not been characterized (Kroes et al., 1999; Aas et al., 2005). These biofilms are responsible

for a plethora of ailments including oral cavities and periodontitis, where some species such as *Porphyromonas gingivalis, Tannerella forsythensis* and *Treponema denticola* have been isolated (Pihlstrom et al., 2005). The establishment of biofilms in the dental cavity has been divided into biofilm on exposed enamel surfaces, and biofilms below the gum line and within the periodontal pocket (Kolenbrander et al., 2010). In these biofilms bacteria start building communities comprised of commensal species, and can change the environment, like decreasing pH conditions. This causes pathogenic strains to multiply, allowing for the initiation of serious conditions such as gingivitis and periodontitis (Kolenbrander et al., 2010). Similar to cystic fibrosis, dental biofilms harbour bacterial species containing mobile genetic elements such as conjugative plasmids, and transposons conferring antibiotic resistance to the biofilm community (Hannan et al., 2010).

Urinary tract infections (UTI) including cystitis and pyelonephritis, where result from certain *E. coli* strains called uropathogenic *E. coli* (UPEC) (Mulvey, 2002). Biofilm formation in these strains has been studied rigorously leading to a correlation between the recurrence of UTI and biofilms (Ulett et al., 2007c). Usually abiotic devices, like catheters, are introduced into patients containing UPEC strains where it is suspected the strains can swim to the kidney and bladder to colonize and invade those tissues (Jacobsen et al., 2008). Once established, intracellular UPEC can form a polysaccharide-like biofilm capsule that may provide some form of protection (Anderson et al., 2010). In addition, other *E. coli* strains have been researched for they ability to form biofilms

including pathogenic strain O15:H7, adherent and invasive *E. coli* (AIEC), and commensal strains like K-12.

E. coli strain O157:H7 is responsible for causing haemorrhagic colitis and haemolytic uremic syndrome in humans, and it is a major food-borne pathogen whose principal reservoir are cattle (Callaway et al., 2009; Nataro and Kaper, 1998). These bacteria colonize the recto-anal junction in the animal via intimin, a virulence factor named Tir, and a plasmid pO157 (Sheng et al., 2006). Due to the presence of these virulence traits, O157:H7 can persist for days in feces after shedding (Callaway et al., 2009). In slaughter plants, O157:H7 can form biofilms on steel surfaces, surviving sanitation procedures such as treatment with organic acids (Skandamis et al., 2009). Recently it has been reported that the bacterium requires the pO157 plasmid in order to produce a normal biofilm, since its absence reduced polysaccharide production and increased viscosity on steel surfaces (Lim et al., 2009). There is a growing concern that antibiotic resistant O157:H7 strains can be isolated from beef cattle (Reinstein et al., 2009), and the fact that the bacterium forms biofilms on abiotic surfaces could become a future threat for human health.

Adherent and invasive *E. coli* (AIEC) represent novel pathotype related to inflammatory bowel diseases, particularly Crohn’s disease (CD) (Rolhion and Darfeuille-michaud, 2007). Evidence demonstrates increased numbers of AIEC strains in CD patients compared to normal control patients (Martinez-Medina et al., 2009; Kotlowski et al., 2007). AIEC are genetically similar to UPEC strains, with the main difference being the absence of toxin-related genes in AIEC (Martinez-Medina et al., 2009_b). In general, a

denser biofilm in the epithelium of CD has been observed with respect to normal patients, in which AIEC have been frequently isolated (De Hertogh et al., 2008; Rolhion and Darfeuille-michaud, 2007). The process by which AIEC attach and invade the epithelium is regulated by attaching organelles like type 1 fimbriae, motility machinery like flagella, and protein membrane receptors such as porins (OmpC) (Carvalho et al., 2009; Claret et al., 2007; Rolhion et al., 2007). Recently it has been shown that flagella and fimbriae contribute to AIEC forming biofilms on plastic surfaces (Martinez-Medina et al., 2009_b).

Despite the importance of biofilms in pathogenic *E. coli*, many investigations were initially performed with commensal K-12 strains (Pratt and Kolter 1998). Strain K-12 biofilms were initially studied with abiotic surfaces like plastic polyvinyl chloride (PVC) 96-well plates, and nutrient poor growth media (Pratt and Kolter, 1998). The aforementioned study indicated that motility was crucial for the initial development of K-12 biofilms, and type 1 fimbriae was required for early attachment. Moreover, motility via flagella had a key role in K-12 biofilm architecture, since mutants lacking in flagella production were unable to fully cover glass surfaces (Wood et al., 2006). Nonetheless many other factors are known to influence the biofilm in this strain, including the temperature at which bacteria are grown (White-Ziegler et al., 2008; Pruss et al., 2006), and the presence of the matrix during biofilm maturation (Danese et al., 2000; Agladze et al., 2005; Itoh et al., 2008). Therefore it has been established that the formation of biofilm involves different developmental stages, starting from the moment when planktonic K-12 swims and attaches to surfaces until it develops rigid colonies (reviewed

by Beloin et al., 2008). This makes for a very complex scenario in terms of physical development and gene expression level within biofilms (Pruss et al., 2006).

In general, bacteria use a communication system enabling them to sense information from their environment, to communicate with each other, and coordinate a response using molecules such as autoinducers and peptides in a process known as quorum sensing (Fuqua et al., 2001). QS controls several bacterial biological functions including biofilm formation (Kaper and Sperandio, 2005). In *E. coli,* studies regarding QS and biofilm formation have been performed with K-12 and O157:H7 strains (Wood, 2009; Parker and Sperandio, 2009). K-12 strains produce a chemical signal derived from methionine metabolism called the autoinducer 2 (AI-2), where the gene *luxS* is involved in its synthesis (Surette and Bassler, 1998; Surette et al., 1999). This bacterium possesses several genes that are responsible for transporting AI-2 into the cytoplasm, initiating a cascade of reactions that controls the expression of genes involved in biofilm formation, like motility-swimming factors. Among the transport-related genes are the operon *lsrACDB* and *lsrK* (Xavier and Bassler, 2005), Once internalized and phosphorylated, AI-2 can regulate the expression of the *qseBC* operon via the motility QS regulator gene (*mqsR*). This in turn regulates the expression of the master flagella regulon genes *flhDC* which is responsible for flagella apparatus synthesis (Gonzalez-Barrios et al., 2006; Sperandio et al., 2002).

Conversely, in O157:H7 the role of AI-2 is uncertain and the regulatory pathways involved in flagella synthesis are governed by another QS system. This is comprised of an unknown aromatic molecule named autoinducer 3 (AI-3), and the mammalian host

catecholamine hormones epinephrine (Epi) and norepinephrine (Ne) which together form the AI-3/Epi/Ne system (Clarke and Sperandio, 2006; Clarke et al., 2006). The three signals can trigger *qseBC* operon expression directly which controls flagella-motility synthesis (Clarke and Sperandio, 2005; Kendall et al., 2007). It has been reported that O157:H7 can sense and utilize AI-3 signals produced autonomously and from the commensal bacterial microbiota in the gut, thus attaching to the epithelium via flagella and fimbria factors regulated by *qseBC* operon (Kendall et al., 2007). In addition, virulence genes such as the locus of enterocyte and effacement (*lee*) can be activated via the *qseEF* sensor system (Walters and Sperandio, 2006; Sharp and Sperandio, 2007, Reading et al., 2009). In this way, O157:H7 releases Shiga_toxins, thus degrading epithelial cells, which leads to contact with the catecholamines Epi and Ne, exacerbating pathogenesis (Hughes et al., 2009).

In this research we initially conducted a general survey of a collection of environmental *E. coli* strains from our laboratory including from cattle, pigs, soil, and humans sources. The objective was to try to correlate the biofilm formation ability under different media conditions, with the presence of genes involved in AI-2 QS and adhesion factors involved in biofilms. Additionally we were seeking to understand the biofilm-quorum sensing phenomenon at the gene level in AIEC. In this case, we decided to construct mutations of selected genes involved in biofilm formation, controlled via QS systems which included the synthesis of AI-2 QS molecules, and two component phosphotransfer systems. Furthermore, we performed microarray experiments using a

static biofilm model to understand the biofilm phenomenon at a global scale, simulating conditions that may occur in the *E. coli* environmental host.

LITERATURE REVIEW

1.0 Initiation and dispersion of biofilms in *Escherichia coli*: the role of swimming motility.

E. coli possesses the ability to swim and swarm. Differences between these concepts rely on the fact that unicellular bacteria swim (a solitary activity), whereas a group of bacteria swarm (a communal activity), with both depending on cell surfaces appendages called flagella (Harshey, 2003). Flagella synthesis and function are dependent on approximately 50 genes, organized in 17 operons divided into three hierarchical categories including class1, class 2 and class 3 genes (reviewed by Soutorina and Bertin, 2003). Class 1 genes includes the *flhDC* master regulon, responsible for activation of class 2 genes. Class 2 genes are responsible for the flagella basal body formation, and involves the *fliA* gene which encode a sigma factor (σ^{28}) that positively regulates the genes included in class 3. This class contains genes encoding proteins that form the flagella filament like flagellin (FliC), as well as motor and chemotaxis proteins (reviewed in Chilcott and Huges, 2000).

Motility gives *E. coli* the ability to swim to surfaces and adhere to structures via flagella, which is the first step in biofilm development (Harshey, 2003; Beloin et al., 2008). Earlier studies determined the ability of *E. coli* K-12 to form biofilms, using plastic surfaces like PVC under diverse nutrient-poor medium growth conditions (Pratt and Kolter, 1998). In addition, isogenic mutations of genes involved in flagella synthesis,

showed a decreased ability to form a biofilm by the mutants. Moreover, it was established that *E. coli* K-12 uses the flagella apparatus to define its architecture in the biofilm, since mutants deficient in the ability to swim form flatter biofilms compared to wild type (Wood et al., 2006). However the importance of motility in biofilm formation is not universal in *E. coli* (Reisner et al., 2006).

A study reported that a shiga-producing strain *E. coli* O157:H$^-$ is non-motile due to a 12 base-pair nucleotide frame-shift mutation in the *flhC* gene. The region codes for the amino acid phenylalanine impeding normal O157:H7 flagella synthesis without affecting its virulence capability (Monday et al., 2004). In addition, an *in vivo* experiment in beef steers demonstrated the importance of the *flhDC* operon on the gut colonization by O157:H7 as a mutation conducted in the *flhC* gene resulted in lower colonization ability (Dobbin et al., 2006). Given that *flhDC* expression is a crucial factor for flagella synthesis, it is considered a key virulence factor that allows O157:H7 to be motile. It also functions as an adhesin enhancing attachment to mucins which are the main proteins covering the epithelium of the gastrointestinal tract (Mahajan et al., 2009). Furthermore, it is known that *E. coli* O157:H7 is able to survive the acidic stomach conditions by increasing its motility (House et al., 2009).

Uropathogenic *E. coli* (UPEC) can use motility to approach a surface, playing an important role in its adherence to urinary tract epithelial surfaces (Yamamoto et al., 1990). However, the precise role of flagella in UPEC infection is not well understood. It has been suggested that the ability to be motile is key to allowing UPEC to swim from the bladder to the kidney (Lane et al., 2007a). Moreover there is evidence showing that

the FliC flagella protein is required by UPEC to invade renal cells in a mouse model (Pichon et al., 2009). As UPEC approaches an epithelial surface and begins attachment, motility-flagella associated gene synthesis is decreased because of the production of fimbria. It seems that a reduction in motility induces the synthesis of several fimbria including type 1, as well as pap that anchors UPEC to specific receptors on epithelial cells (Lane et al., 2007b; Simms and Mobley, 2008_a; 2008_b). On catheter surfaces, UPEC starts colonizing via motility and later anchors itself with fimbria to the catheter surface, initiating the biofilm which is the cause of many recurrent infections (Jacobsen et al., 2008).

Adherent and invasive *E. coli* (AIEC) strains have been implicated in inflammatory bowel diseases (IBD), and in particular Crohn's Disease (CD) (Rolhion and Darfeuille-Michaud, 2007; Sartor, 2008). Typically, the flagellar apparatus is related to the pathogenesis in this type of *E. coli* whereby flagella is considered a virulence factor involved with AIEC adherence and invasion in *in-vitro* and *in-vivo* models (Barnich et al., 2003; Carvalho et al., 2008). Studies have reported that AIEC mutant strains incapable of synthesizing flagella are unable to attach and invade cell lines (Barnich et al., 2003). The absence of sigma factor FliA and transcriptional regulator FlhDC, which are involved with flagella synthesis, results in a reduction of adherence and invasion (Claret et al., 2007). However, the AIEC adherence and invasion mechanisms do not depend exclusively on the flagella apparatus. Other factors such as fimbria or adhesins can target specific receptors on host epithelial surfaces (Barnich et al., 2007).

2.0 Adhesion to a surface: the role of fimbriae or adhesins in *E. coli* biofilms.

E. coli uses pili or fimbriae to attach to surfaces (Knight and Bouckeart, 2009). *E. coli* can contain up to 500 fimbriae and carbohydrate-binding two domain adhesions (TDA) are expressed at the tip of many fimbriae. Those TDA can include type 1, P and S fimbriae that have the ability to bind mannose, Galα1-4Gal, and α-sialic acid receptors respectively (Knight and Bouckeart, 2009; Balsalobre et al., 2003).

Type 1 fimbriae are between 1-2 μM long and 7 nm wide and bind to N-mannose. Synthesis of this organelle involves eight genes encoding proteins for synthesis, including the FimA, FimF, FimG and FimH subunits which form the main body. FimC and FimD are involved with fimbria assembly, and FimB and FimE regulate fimbrial expression (Knight and Bouckeart, 2009). P fimbria binds to Gal(α1-4)Gal receptors on the host epithelial cells. It is synthesized by up to 11 pap (pyelonephritis-associated pili) genes, including *papA* that is involved in the synthesis of the rigid fimbria stalk body, the *papE* and *papF* genes that are related to minor subunit proteins, and *papG* that encodes the tip receptor binding protein PapG (Lane et al., 2007a). S fimbria comprises the S fimbrial adhesin (SfaI) that attaches to α-sialyl-2,3-β-Gal protein receptors on epithelial and endothelial cells (Khan et al., 2000). There are four proteins that comprise the S fimbria with SfaI-A as the major subunit, and SfaI-G, SfaI-S and SfaI-H comprising the minor protein subunits. The SfaI-S subunit has been determined to be the primary binding receptor of host cells (Khan et al., 2000).

2.1 Role of different fimbriae on diverse *E. coli* strain biofilm formation.

Commensal K-12 strains attach to polyvinyl chloride surfaces by means of type I fimbriae (Pratt and Kolter, 1998). Mutations of the *fimA* and *fimH* genes lead to a reduction in initial attachment and it was demonstrated that expression of *fimA* and *fimF* was significantly increased in the biofilm stage compared to the planktonic stage (Schembri et al., 2003). Additionally, when a mutant strain of the *fimA* gene was challenged with bacteriophages, it became totally susceptible upon the bacteriophage exposure. However, the parental strain seemed to increase biofilm formation in the presence of bacteriophages (Lacqua et al., 2006). This evidence indicates the importance of type 1 fimbriae in initial biofilm formation by K-12.

Studies conducted on UPEC indicate that type 1 fimbria can also initiate biofilm formation on plastic and glass surfaces, as well as cell tissues under *in-vivo* conditions (Pratt and Kolter, 1998; Bahrani-Mougeot et al., 2002). However, the adhesin portion of type 1 fimbria may not be completely necessary for biofilm formation (Orndoff et al., 2004). Indeed, once *E. coli* is attached there has to be an interaction between individual cells, a process known as auto-aggregation, in order to develop a biofilm. This auto-aggregation occurs via antigen 43 (Ag43), which promotes biofilm growth and allows UPEC to remain in the urinary bladder for the long term (Ulett et al., 2007). Furthermore, in AIEC strains, which are genetically related to UPEC, type 1 fimbria mediate adherence to and invasion of epithelial cells (Barnich et al., 2003). In particular, this process involves the FimH adhesin that recognises a protein receptor in Crohn's Disease patients referred to as carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6). It

allows AIEC to adhere and invade the gut epithelial cells (Barnich et al., 2007). Nonetheless there are no reports indicating the precise role of type 1 fimbria on AIEC biofilm formation under abiotic conditions. Conversely, the role of type 1 fimbria in O157:H7 shiga toxin-producing strains (STEC) during biofilm formation is not well understood. Shiga toxing-producing *E. coli* attach to epithelial cells using a type III secretion system which is regulated by the locus of enterocyte and effacement (LEE) (Nataro and Kaper, 1998). Nevertheless, on abiotic surfaces this strain produces a weak biofilm; explanation for this phenomenon is not clear (Cookson et al., 2002; Ulich et al., 2002).

P and S fimbriae organelles have been studied mainly in UPEC and meningitis-associated *E. coli* strains. It seems that they are not present in commensal strains (Schmoll et al., 1989; Khan et al., 2000; Dobrindt et al., 2001). Importantly, there is evidence associating the presence of P and S fimbriae with the development of urinary tract infections, meningitis, and biofilms by both *E. coli* strains (Holden and Gally, 2004; Lane et al., 2007a; Naves et al., 2008). However, there is no clarity as to what exactly their role are in pathogenesis and biofilm formation. For instance, Naves et al., (2008) conducted a survey study and correlated several fimbriae genes with biofilm formation in *E. coli* from urinary tract infections. Interestingly, it was reported that strong biofilm forming strains had both *pap* and *sfa* fimbriae genes. Moreover, there are studies indicating that both fimbriae are expressed by UPEC during kidney infections (Holden and Gally, 2004; Lane et al., 2007b). Recently, Melican et al., (2011) suggested a synergy between P and type 1 fimbria where P fimbria promotes early attachment to the renal

tubules while type 1 fimbria mediates adhesion. It was also reported that biofilm formation on plastic surfaces was reduced once a mutation of the *fimH* gene was generated, but no reduction in biofilm formation was observed in a *papG* mutant.

In meningitis-related *E. coli* strains, studies have found that the SfaI-S minor subunit is responsible for modulating specific adhesion to the sialic acid receptors epithelial cells and it is needed for proper S fimbria development (Khan et al., 2000). Moreover, Sjostrom et al., (2009) reported a new genetic component of the S fimbria named SfaX that seems to play a role in motility regulation in this *E. coli* strain. It was also reported that the flagellar and type 1 fimbria genes were down regulated upon expression of *sfaX*, indicating a role for S fimbria in meningitis-related *E. coli* adhesion (Sjostrom et al., 2009). Nevertheless the effects of *sfaX* on biofilm formation was not evaluated, thus leaving no clues as to what extent these fimbriae can affect the biofilm. In addition, no studies have been conducted in other pathogenic strains such as AIEC to evaluate the role of these fimbriae organelles in biofilm formation.

3.0 The two component system EnvZ/OmpR and its role in the establishment of colonies in *E. coli* strains.

Porin proteins are localized in the outer membrane of *E. coli* and are regulated in response to changes in osmolarity in the surrounding environment. These proteins provide *E. coli* with the ability to activate or inactivate specific genes depending whether the bacterium is inside or outside the gut system (Reviewed in Pratt and Shilhavy, 1995; Pruss et al., 2006). Initial studies conducted on the *E. coli* K-12 strain revealed a two component system comprised of the proteins, EnvZ and OmpR which are involved in the

regulation of porins expression, including the outer membrane proteins OmpC and OmpF (Pratt and Shilhavy, 1995).

Two-component systems comprise a transmembrane sensor protein (SP) that can detect the external environment, and a cognate receptor regulator (RR) that is phosphorylated once the SP is activated. EnvZ is a SP while OmpR is a RR (Pratt and Shilhavy, 1995). The EnvZ protein senses osmolarity changes externally, caused by salts (NaCl), sugars (arabinose) and other molecules like alcohols (Clarke and Voigt, 2011). The EnvZ protein has an N-terminal peptide that extends into the periplasmic region that receives the osmolarity stimuli, and passes it to the C-terminal portion at a histidine residue where autophosphorylation occurs (Pratt and Shilhavy, 1995). Then the phosphorylated EnvZ either transfers or removes the phosphate from OmpR using kinase and phosphatase enzymes respectively, and depending on the osmolarity signal received, will up-regulate expression of either OmpF or OmpC porins (Pratt and Shilhavy, 1995). At low osmolarity, for example, OmpF will be activated whereas at high osmolarity, OmpF will be repressed and OmpC will be activated (Pratt and Shilhavy, 1995; Pruss et al., 2006).

In addition to regulating OmpF and OmpC, the RR OmpR can affect the expression of other biological processes like fimbriae and flagella activity (Shin and Park, 1995; Pringent-Combaret et al., 2001). In the *E. coli* K-12 strain it has been shown that OmpR can directly interact with the master flagellar regulon *flhDC* (Shin and Park, 1995). Using Dnase I foot printing, two sites in the promoter region of *flhDC* were identified the sites of interaction with OmpR. It was also demonstrated that the *flhDC*

regulation was dependent on phosphorylated OmpR having greater affinity for the *flhDC* promoter region than the non-phosphorylated form of OmpR (Shin and Park, 1995). Oshima et al., (2002) constructed several mutations of different two component systems in *E. coli* K-12, and found that a mutation in the EnvZ/OmpR system affected 125 genes, among which flagellar related genes were up-regulated and the mutants were more motile than the parental strain. Recently it has been suggested that while EnvZ/OmpR affects the expression of flagella, it can promote the expression of fimbria organelles like curli (Pringent-Combaret et al., 2001), thus promoting the formation of biofilm in K-12 (Pruss et al., 2006).

The role of the protein OmpR in pathogenic *E. coli* such as UPEC and AIEC is more pronounced and differs from K-12 (Rolhion et al., 2007; Shaw, 2009). In AIEC strain LF82 it was found that OmpR is an essential factor that regulates not only the expression of porins, but also it affects the expression of flagella and type 1 pili (Rolhion et al., 2007). This study used a mutation in the *ompR* gene, and reported that the mutant was unable to attach and invade cell lines and did not express flagella and type 1 pili. This is in contrast with the results obtained in the K-12 strain where an *ompR* mutant over-expressed the flagella genes (Rolhion et al., 2007). A study performed on a UPEC strain with a mutation in the *ompR* gene had a decreased ability to grow under high concentrations of salts and sugar (> 400mM of NaCl and sucrose). Also, it was shown that the *ompR* mutant significantly decreased the ability to infect the urinary tract of mice (Shwan, 2009). All of this evidence indicates that this two component system is crucial

for the virulence of pathogenic *E. coli*, but no reports have indicated the role of EnvZ/OmpR in biofilm formation in those pathogenic strains.

4.0 Cellulose synthesis and role in biofilm formation by *E. coli*.

Cellulose is a polysaccharide consisting of glucose molecules linked by 1-4β glycosidic bonds, and its production by bacteria can be detected using dyes such as congo-red or calcoflour (Romling, 2002). In the Enterobacteriaceae family, cellulose synthesis is accomplished via the *bcs* operon whereby the *bcsA* gene encodes the cellulose synthase protein, and *bcsB* codes for the c-di-GMP binding protein (Romling, 2002). Cellulose comprises one component of the biofilm matrix (Beloin and Ghigo, 2008). Cellulose production and function varies within *E. coli* strains. For example, the role of cellulose in *E. coli* strain K-12 MG1655 has been linked with resistance to environmental stress rather than biofilm formation (Gualdi et al., 2008). In contrast, studies conducted on commensal *E. coli* strains isolated from the gut of healthy individuals revealed that cellulose is required for biofilm formation (Da Re and Ghigo, 2006), and it is important for colonization in the gastrointestinal tract (Monteiro et al., 2009). Also, pathogenic strains like *E. coli* O157:H7 may use cellulose to adhere to human and bovine cells (Saldaña et al., 2009).

The lab *E. coli* K-12 strain requires a temperature of 30°C before cellulose is synthesized, but a physiological temperature of 37 ^{o}C inhibits its production (Gualdi et al., 2008). It also seems that cellulose does not enhance biofilm formation in this strain. These authors showed that deleting *bcsA*, which is responsible for cellulose synthesis, had a limited effect on biofilm formation either at 30 ^{o}C or 37 ^{o}C (Gualdi et al., 2008).

Conversely, in a commensal *E. coli* isolated from the human gut, it was demonstrated that the ability to form biofilms can be lost once a mutation occurs in the *bcsC* gene. This mutant also lost its capacity to aggregate at 37 ^{o}C, suggesting that cellulose is necessary for the production of the biofilm (Da Re and Ghigo, 2006). Additionally, in *E. coli* Nissle 1917 it was determined that cellulose played a significant role in adherence under *in-vitro* and *in-vivo* conditions (Monteiro et al., 2009). A mutation in the *bcsA* gene dramatically reduced *E. coli* Nissle's adherence to the cell lines and the epithelial lining in the mouse gut (Monteiro et al., 2009). This evidence indicates that the role of cellulose is different among commensal *E. coli*.

Notwithstanding the positive relationship between biofilm formation and cellulose synthesis in commensal *E. coli*, the role of cellulose synthesis in biofilm formation in pathogenic strains is not well understood. Bokranz et al., (2005) conducted a survey study of the ability of urinary tract infection (UTI) *E. coli* to form biofilm and produce cellulose. This study reported that UTI strains had a low capacity to form biofilm in either LB or M9 medium, and only a few strains were capable of producing cellulose at 37 ^{o}C. Wang et al., (2009) studied *E. coli* isolated from catheters and urine and reported that there was not a significant relationship between biofilm formation and cellulose production, even though there was one particular strain that had high biofilm formation ability and high levels of cellulose expression. In *E. coli* O157:H7 strains it is not known specifically how cellulose affects biofilm formation, but there is evidence showing that cellulose is involved in adherence to human and bovine digestive tract epithelial cells (Saldaña et al., 2009). In addition, it was determined that the adherence to the gut is not

determined by cellulose alone and other adherence factors are critical. In fact, it was demonstrated that curly fimbria play a significant role in this process. This indicates that a level of synergy exists between cellulose production and curly fibres, and that they do not act independently of each other during the adherence process (Saldaña et al., 2009).

4.1 Regulation of cellulose synthesis in *E. coli*

Cellulose synthesis regulation is a very complex phenomenon. In enterobacteria like *Salmonella* for example, this phenomenon involves a AgfD-regulated protein (AdrA) that contains four N-terminal units of the GGDEF domain. These domains may be involved in the regulation of a second messenger molecule, called cyclic di-guanosine mono phosphate (c-di-GMP) (Romling, 2002). The interaction between the GGDEF domain of AdrA and the c-di-GMP molecule could initiate cellulose production (Romling, 2002). In *E. coli*, up to 19 GGDEF domains working at the post-transcriptional level have been identified (Romling et al., 2005). It has been shown that AdrA is not the only protein involved in the regulatory cascade of cellulose synthesis. Indeed, the YedQ protein contains a GGDEF domain located at the C-terminal portion that has c-di-GMP synthase activity. It was demonstrated that this protein is involved in the regulation of cellulose production in a commensal *E. coli* strain (Da Re and Ghigo, 2006). Recently LeQuere and Ghigo (2009) discovered a new gene (*bcsQ*) localized upstream of the *bcs* operon which could also be involved in the regulation of cellulose synthesis. Overall, it is safe to say that the regulation of cellulose synthesis and its role in biofilm by *E. coli* is a developing area.

In summary, it can be demonstrated that biofilm formation is a very complex phenomenon particularly at the gene expression level. The biofilm formation process in *E. coli* consists of several stages that include: 1) arrival at a surface using swimming organelles like flagella, 2) anchoring to a surface or irreversible attachment that can involve more than one cellular appendage called fimbria, 3) establishment of colonies that require multiple pathways, one of which includes the EnvZ/OmpR two-component system, and 4) formation of the matrix which is composed of several polysaccharides including cellulose; its expression is suspected to be controlled by a second messenger molecule called cyclic-di-GMP. In addition, there is an increasing amount of information indicating that between and within biofilms, other molecules can be used by bacteria that influence several stages in biofilm formation. The mechanism by which bacteria recognize and use these molecules is usually referred as quorum sensing.

5.0 Quorum sensing: a process of communication among bacteria.

The discovery of what is known as QS occurred between 1960 and 1970. It brought about the idea that bacteria use complex communication systems to coordinate responses to environmental stimuli (Bassler and Losick 2006). A quorum-sensing system enables bacteria to sense, integrate, and process information from their environment, communicate with each other, and coordinate responses within a bacterial population by means of chemical molecules known as autoinducers (Fuqua et al. 2001).

Bacteria use QS to modulate gene expression, thus controlling a variety of cellular processes such as biofilm formation, light production, and virulence factors in different environments like the gastrointestinal tract, rhizosphere or sea water (Kaper and

Sperandio 2005, Visick and McFall-Ngai 2000). Since the first reports of QS reported in *Vibrio* species from marine sources (Bassler and Losick 2006). Many investigations have focused on pathogenic bacteria that can affect humans, animals and plants (Kaper and Sperandio 2005, Cui and Harling 2005).

5.1 The autoinducer 2 (AI-2) quorum-sensing system is a "universal" language for bacterial communication.

5.2 The autoinducer 2 (AI-2) system in *Vibrio harveyi*.

Recent publications have indicated that the free-living marine bacterium, *Vibrio harveyi,* contains not one but three distinct signal molecules (Waters and Bassler 2006, Duan and Surette 2006). Similar to other Gram-negative bacteria, *V. harveyi* produces an acylated homoserine lactone signal molecule, plus a molecule of unknown structure called CAI-1 and a furanosyl borate diester denoted as AI-2 (reviewed in Xavier and Bassler 2003, Duan and Surette 2006, Miller and Bassler 2001).

In a similar manner to *V. fischeri, V. harveyi* regulates the production of light through QS. At least two systems are involved in the regulation of the luciferase operon, *luxCDABE.* System I, or autoinducer I (HAI-1) consists of a N-(3-hydroxybutanoyl)-homoserine lactone. Interestingly, this HAI-1 molecule is not synthesized by a LuxI-protein as in *V. fischeri*. Instead, synthesis of this homoserine lactone signal is dependent on a LuxLM protein, which does not have significant homology to LuxI (Miller and Bassler 2001, Duan and Surette 2006). The second signal molecule involved in luciferase regulation is AI-2. Synthesis of this autoinducer requires two proteins, Pfs and LuxS, the

latter is encoded by the *luxS* gene. The synthesized AI-2 signal is a furanosyl-borate-diester, a molecule that contains boron in its structure. It is noteworthy that *luxS* homologues are present in almost half of all completed bacterial genomes that have been sequenced (Xavier and Bassler 2003). Bacterial species that produce LuxS include: *Escherichia coli, Salmonella spp, Haemophilus influenzae, Helicobacter pylori, Bacillus subtilis, Borrelia burgdorferi, Neisseria meningitidis, Yersinia pestis, Campylobacter jejuni, Vibrio cholerae, Mycobacterium tuberculosis, Enterococcus faecalis, Streptococcus spp, Staphylococcus aereus, Clostridium perfringers, C. difficile, and Klebsiella pneumoniae* (Miller and Bassler 2001). Additionally, *luxS* has been reported in bacterial species isolated from complex environments such as the rumen of cattle. Examples include *Ruminococcus albus, R. flavefaciens, Selenomonas ruminantium* and *Prevotella ruminicola* (Mitsumori et al. 2003).

Surette and Bassler (1998) demonstrated that many bacterial species, when tested for AI-2 production using a *V. harveyi* detection assay, did not produce acyl-homoserine-like signal (HAI-1). Given the presence of *luxS* in diverse bacterial species and the fact that the AI-2 signal produced by different bacteria could be detected in a *V. harveyi* model, it was hypothesized that AI-2 is a universal signal used for interspecies communication (Xavier and Bassler 2003, Surette and Bassler 1998, Surette et al. 1999). On the contrary, the autoinducer 1 signal (HAI-1) of *V. harveyi* is used for intraspecies communication because HAI-1 was proven to be produced exclusively by *V. harveyi* and *V. parahaemolyticus*. Finally, the third signal discovered in *V. harveyi*, CAI-1, has been

suggested to act as an intergenera signal due to the fact that it is only produced by the genus *Vibrio* (Waters and Bassler 2006).

Earlier studies described a circuit in *V. harveyi* whereby only HAI-1 and AI-2 were involved in the expression of bioluminescence (Xavier and Bassler 2003, Miller and Bassler 2001, Shauder et al. 2001). In these earlier studies, it was noted that the circuit consisted of the cognate signal sensors LuxN (for HAI-1), and LuxQ (for AI-2). In addition, the AI-2 system requires the protein LuxP to transport the AI-2 signal. Once the signal is transported, LuxN and LuxQ follow a series of phosphorylation steps, culminating in adherence to a signal integrator protein LuxU, which is responsible for transferring the phosphate-bound signal to the RR, LuxO. LuxO indirectly represses the expression of the *luxCDABEGH* operon. A recent study carried out by Waters and Bassler (2006), revealed a novel model of gene regulation by the three different quorum-sensing signals in *V. harveyi*, indicating that regulation of bioluminescence is more complex than previously thought. In this case, the third signal, CAI-1, is added to the circuit with its respective sensor protein CqsS. Together, the three sensor systems, LuxN (HAI-1), LuxQ (AI-2), and CqsS (CAI-1), converge and transfer a phosphate to the integrator protein LuxU, which in turn transfers the phosphate to LuxO. The bound phosphoryl-LuxO binds to a sigma (σ54) activator, which activates the expression of genes encoding five small RNAs called Qrrl-5. Together the Qrrl-5 RNAs with a chaperone, Hfq, destabilizes the *luxR*_mRNA which encodes the master transcriptional activator LuxR, which is dissimilar to the LuxR family protein from *V. fischeri*. In turn,

LuxR regulates the expression of different genes responsible for diverse functions such as bioluminescence, biofilm formation and protease production (Waters and Bassler 2006).

5.2.1 Metabolic pathways and kinetics of the AI-2 system.

The production of AI-2 in *V. harveyi* starts from the metabolite S-adenosylmethionine (SAM), a molecule derived from the amino acid methionine. This molecule is used as a methyl donor to produce S-adenosylhomocysteine (SAH) which may be toxic to the bacterium. The removal of SAH is carried out by a nucleosidase, Pfs (S-adenosylhomocysteine/5'-methylthioadenosine), generating S-ribosylhomocysteine and adenine as products. Then LuxS catalyzes the convertion of S-ribosylhomocysteine to produce 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine (Shauder et al. 2001). In theory, once DPD is produced it can be converted into a complex of furanone rings, and one of them leads to the formation of the AI-2 molecule, which is apparently very unstable. Initially scientists had difficulties unraveling the structure of AI-2, but finally it was determine that AI-2 is a furanosyl borate diester. In essence, the discovery of a boron atom in the *V. harveyi* AI-2 structure was a surprise due to the lack of knowledge about the role of boron in biological systems (Xavier and Bassler 2003).

To date, the synthetic pathway of DPD has been demonstrated to be identical in two other bacterial species; *E. coli* and *S. typhimurium* (Xavier and Bassler 2003, Duan and Surette 2006). Despite the fact that DPD production is the same in *V. harveyi*, *E. coli* and *S. typhimurium*, the chemical structure of AI-2 varies between species. For example, *V. harveyi* possesses an AI-2-borate structure: (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (S-THMF-borate). In contrast, the *S. typhimurium* AI-2

signal does not contain borate in its structure: (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (S-THMF). To date, the structure of the AI-2 molecules has only been deduced for those two bacterial species (Duan and Surette 2006).

5.3 The autoinducer 2 (AI-2) quorum-sensing system in *E. coli*.

Early studies demonstrated the presence of the AI-2 system in *E. coli* (Surette and Bassler 1998, Surette et al. 1999). In these reports, the presence of the AI-2 signal was detected using a *V. harveyi* bioassay. The reports showed that *E. coli* was able to secrete an AI-2 signal capable of inducing light production in *V. harveyi*. It was also noted that: 1) AI-2 signal was not produced in the absence of glucose; 2) the signal was maximally produced at the mid-exponential phase of growth, and declined during stationary phase. Later on, DeLisa et al. (2001 b), reported that in nonpathogenic *E. coli* strains, AI-2 production was stimulated using glucose, iron and NaCl. Also, AI-2 was produced in LB medium with or without glucose supplementation, indicating that glucose can increase AI-2 production but is not essential for the synthesis of the signal. Moreover, it was noted that intracellular and environmental stimuli can affect AI-2 (DeLisa et al. 2001b). For example, increasing iron and osmolarity, and decreasing the redox potential in the culture media led to a large and transient AI-2 accumulation. In contrast, exposure to heat shock, ethanol, H_2O_2, sodium acetate and oxygen seemed to decrease AI-2 activity as well as increase its degradation (DeLisa et al. 2001b).

Sequence analysis of the gene responsible for AI-2 production (*luxS*) showed a high degree of similarity between *E. coli* strains (K-12, O157:H7) and *V. harveyi* (Surette et al. 1999). Nevertheless, the transport system of AI-2 is very different between these

two bacterial species. In *V. harveyi*, the LuxP protein is responsible for transporting the signal and initiating the signal cascade. This system has only been found in *Vibrio* species (Waters and Bassler 2006). On the other hand, in *E. coli* the AI-2 signal regulates a group of genes called lsr (LuxS-regulated) which include a series of transporters, named ACDB and K (Xavier and Bassler 2005). This lsr system is responsible for AI-2 uptake, whereby AI-2 binds to LsrB and the signal is transported inside the cell. Then LsrK phosphorylates the AI-2 signal (Xavier and Bassler 2005). Despite these studies, the role of AI-2 and how it is involved in other metabolic processes is not clear. Many studies have indicated that AI-2 can control several cellular processes including cell division and biofilm formation, particularly in commensal *E. coli* strains (K-12) (Delisa et al. 2001a,b, Ren et al. 2004, Domka et al. 2006, Gonzalez-Barrios et al. 2006). In pathogenic strains (O157:H7) the role of AI-2 is uncertain. Nonetheless there is evidence that virulence factors of O157:H7 are controlled by another quorum-sensing system involving the signal molecule AI-3 (Clarke and Sperandio 2006).

Recent studies in *E. coli* K-12 have shown that AI-2 is involved in biofilm formation. Initially, Delisa et al. (2001a) carried out a microarray experiment using an *E. coli luxS* mutant where it was proven that the mutant was unable to synthesize AI-2. It was demonstrated that approximately 5.6% of the *E. coli* genome was affected (genes repressed or induced) by the presence of the AI-2 signal. Moreover it was noted that a group of 23 genes regulated by AI-2 were involved in biofilm formation. In a recent study, Gonzalez-Barrios et al. (2006) reported that AI-2 induces biofilm formation in *E. coli* K-12 via *mqsR*, which is a mediator between AI-2 and the QS regulon, *qseBC,* which

is responsible for bacterial motility. This study used microarray and *in-vitro* biofilm experiments in two different media, one rich (LB) and one minimal (M9). The authors proposed a model whereby AI-2 enhances biofilm formation by stimulating expression of *mqsR*. Consequently MqsR induces expression of the *qseBC* regulon, which in turn promotes motility by using the master regulon FlhDC, leading to biofilm formation (Gonzalez-Barrios et al. 2006).

Domka et al. (2006), found that the proteins YliH and YceP in *E. coli* K-12 are also involved in biofilm formation. Based on point mutations and microarray analysis, it was found that these two proteins are capable of regulating several cellular functions including cell motility. Moreover, it was reported that mutations of *yliH* and *yceP* increased extracellular AI-2 concentrations and this induced biofilm formation. However, the precise mechanism of how this occurr was not clear (Domka et al. 2006). In a recent study by Herzberg et al. (2006), the presence of *ydgG* mutants resulted in increased biofilm. The results of this investigation suggested that *ydgG* may be responsible for controlling transport of AI-2. Furthermore, it was reported that when *ydgG* was deleted, AI-2 intracellular activity increased during both exponential and stationary phases of growth. In this way, a *ydgG* mutation was able to induce genes involved in biofilm formation such as flagellar synthesis (FlhDC), and adhesion (fimbria)-related genes. Nonetheless, the results concerning the AI-2 transport system were not clear enough to define whether *ydgG* is responsible for either import or export of the AI-2 signal (Herzberg et al. 2006).

Despite all of this evidence, there are still gaps in our knowledge of how AI-2 regulates biofilm formation in *E. coli* K-12. To summarize, research has confirmed the role of AI-2 in biofilm development, via studies involving microarray assays and point mutations of specific genes. Nevertheless, the biochemical process of how AI-2 can regulate all of the genes involved in biofilm formation remains unclear. It is noteworthy that only one study using biochemical and molecular techniques showed that AI-2 indeed regulates the genes involved in transporting of the AI-2 signal, and includes the *lsrACDB* operon and *lsrK* (Xavier and Bassler 2005).

5.3.1 Role of autoinducer 3 (AI-3) in the virulence of pathogenic *E. coli*.

Earlier studies involving pathogenic *E. coli* were designed to determine the role of QS in the expression of virulence factors in enterohemorrhagic (EHEC) and enteropathogenic *E. coli* (EPEC) (Sperandio et al. 1999, 2001). Enteropathogenic *E. coli* is responsible for causing watery diarrhoea in children, while EHEC causes bloody diarrhoea, and it is related to hemolytic-uremic syndrome (HUS). The main difference between the two strains is that EHEC produces shiga toxins and the EPEC does not. However, both types can cause intestinal lesions known as attaching and effacing (AE) lesions. A group of genes localized in a pathogenicity island are called the locus of enterocyte effacement (LEE), which is responsible for AE lesions (Kaper and Sperandio 2005). An initial study by Sperandio et al. (1999) demonstrated that the expression of the LEE operons is regulated by QS in EPEC and O157:H7. Through the use of *luxS* mutants and *lacZ* reporter gene fusions, it was determined that manipulation of the *luxS* gene affected activation of the LEE operons. Therefore, it was believed that AI-2 signal was

essential for transcription of LEE. However, later experiments using microarray assays found that AI-2 was able to regulate between 6 and 10% of the *E. coli* genome (Delisa et al. 2001a, Sperandio et al. 1999). This large number of genes regulated by AI-2 led to the hyothesis that AI-2 had pleiotropic effects. In fact, *luxS* is responsible for catabolizing ribosyl-homocysteine into DPD, which is required for production of AI-2. This pathway is interrupted by mutations in *luxS*; thus changing the overall metabolism of the bacterium (Clarke and Sperandio 2006).

In addition, Kaper and Sperandio (2005), reported that *in-vitro* synthesized AI-2 added to the culture was unable to restore flagellum production and motility in O157:H7 mutants. These two conditions are involved in *E. coli* pathogenesis since it was found that regulation of flagellar expression and motility can be controlled by a quorum-sensing signal mechanism (Clarke and Sperandio 2006). Thus, it was hypothesized that another autoinducer signal must be responsible for controlling virulence factors (LEE operons, flagella and motility). Kaper and Sperandio (2005) reported that AI-2 is a furanone molecule and does not bind to a C18 column. However, they showed that the signal responsible for controlling the virulence factors, binds to C18 column and when eluted with methanol and added to culture will restore motility. This new autoinducer was called AI-3. Additionally, an electrospray mass spectrometry analysis of AI-3 showed a major mass peak at 213.1 Da and minor peaks at 109.1, 164.9, 176.1, 196.1, 211.1, 214.1, and 222.9 Da all of which are different from AI-2 (Kaper and Sperandio 2005). Recently, Walters et al. (2006) reported that AI-3 signal production is affected by the *luxS* gene, although the synthesis of the signal is not dependent on *luxS*. Thus the pathway for AI-3

synthesis and identification of the molecular structure of the signal still remains uncertain (Clarke and Sperandio 2006).

It is important to note that both pathogenic and nonpathogenic *E. coli* strains from the gut are capable of producing the AI-3 signal (Walters et al. 2006). In particular, it is hypothesized that the AI-3 system might be used by pathogenic strains, like O157:H7, to alert the bacterium when it has arrived in the large intestine so that it may undergo activation of virulence genes (Clarke and Sperandio 2006). Clarke and Sperandio (2006), proposed a model in which the AI-3 system of *E. coli* O157:H7 is able to communicate cell-to-cell and between the bacterium and the host. In this case, it has been shown that the AI-3 signal interacts with the intestinal epithelial cells through the catecholamine hormones, epinephrine and norephinephrine. This was determined by using a *luxS* mutant in an *in-vitro* assay, whereby it was found that the *luxS* mutant (no production of AI-3) was still able to produce AE lesions. Later on, using purified catecholamines, it was found that a *luxS* mutant can respond to these molecules (Clarke and Sperandio 2006).

5.3.1.2 The autoinducer-3/epinephrine/norepinephrine (AI-3/Epi/Ne) system coordination of flagella-motility genes using the two component system *qseBC* in *E. coli*.

An earlier investigation conducted by Sperandio et al. (2002) revealed a two-component system named QseBC. This system is made up of a membrane spanning sensor, QseC, and a RR QseB. The QseC sensor kinase contains two domains located between the periplasmic region, a histidine kinase domain in the cytosol, and an ATPase domain that may be responsible for transferring the phosphate group to QseB (Clarke and

Sperandio, 2005a). The input signals sensed by QseC are comprised of an aromatic molecule named autoinducer 3 (AI-3), and the mammalian cathecholamine hormones epinephrine and norepinephrine (Clarke et al., 2006). These signals are received at the trans-membrane domains and transferred to the histidine kinase residue where autophosphorylation occurs. Then the phosphate group is transferred to its cognate response regulator QseB where it coordinates several virulence factors like flagella synthesis (Clarke et al., 2006). Initially the suspicion was that *qseBC* regulates the flagella-motility network in *E. coli* and Sperandio et al. (2002) demonstrated that a mutation in the *qseC* gene decreased motility and flagella activity. Therefore, it was suggested that this two-component system played a role in the flagella-motility network via the flagella master regulator FlhDC (Sperandio et al., 2002). Indeed, activation of *flhDC* depends on the phosphorylated QseB (Clarke and Sperandio, 2005b). The phosphorylated QseB protein binds to two different regions of the *flhDC* promoter: the proximal region (-300bp to +50bp) and a distal region (-900bp to -650bp). The phospho-QseB binds first to the distal region, then later to the proximal region (Clarke and Sperandio, 2005b). In order for *flhDC* transcription to occur, there must be coordinated process which depends on the signal received by QseC. If there is a low input signal then QseB will not be phosphorylated and the protein will bind to a region between -650 and -300 bp which may result in flagella repression. At high levels of input signal, a phosphorylated QseB will bind to both the distal and proximal regions of the *flhDC* operon and flagella is activated (Hughes et al., 2009).

In summary, quorum sensing (QS) is a mechanism of bacterial communication using a plethora of chemical signals, and the QS synthesis and sensory system varies between and within bacterial species. In Gram-negative bacteria such as *V. harveyi* and *E. coli,* a common QS system has been elucidated and is referred to as autoinducer 2 (AI-2). The regulatory role of AI-2 is very complex and depends on the bacterial strain within species. In *V. harveyi,* AI-2 is involved in the regulation of light synthesis and the mechanism of action has been largely resolved. On the other hand, in *E. coli* the results are conflicting. In non-pathogenic strains like K-12, there is evidence that AI-2 has a regulatory role over the flagella-motility network and by default it seems to affect the ability to form biofilm. However, in pathogenic *E. coli* strains, particularly O157:H7, the results indicate that AI-2 does not have a regulatory role in the flagella-motility network. In fact, another QS system has been discovered that allows O157:H7 to cross communicate with the mammalian host and it can activate several genes, including those which regulate flagella-motility genes. The new system is composed of three signals: autoinducer 3(AI-3), epinephrine (Epi) and norepinephrine (Ne) and is called the AI-3/Epi/Ne QS system.

6. Inflammatory bowel diseases (IBD) and pathogenic *E. coli*: the influence of adherent and invasive *E. coli* (AIEC).

6.1 The complexity of IBD.

Inflammatory bowel diseases are composed of several enteric disorders which include Crohn’s disease (CD) and ulcerative colitis (UC). Although a specific etiologic agent is unknown, multiple factors can account for the genesis of these disorders

(reviewed by Sartor, 2008). The factors involved in the pathogenesis of IBD include: I) environmental cues that could affect a susceptible host, II) host genetic immune defects, and; III) microbial pathogens that produce intestinal inflammation (Barnich and Dafeuille-Michaud, 2007; Ogura et al., 2001; Barnich et al., 2007; Graff et al., 2009).

Presently, there are no conclusive studies that have determined which environmental factors affect IBD patients. Nonetheless, there are many investigations that have linked IBD occurrence in a genetically predisposed host. Initially, Tysk et al., (1988), conducted a study in Sweden which determined that heredity could be an aetiological factor in IBD, and appeared to be more important in CD development compared to UC. Moreover, Orhlom et al., (1991) conducted a survey-based study, reporting that first degree relatives of IBD suffers had a 10-fold increased risk of developing UC or CD. Another survey conducted in Denmark using twins reported a high incidence of IBD in which from 103 twin pairs, at least one twin had IBD (Orholm et al., 2000). This study also demonstrated that smoking habits may influence the frequency of IBD occurrence, as the number of cases found in smokers was higher, and even more so in twins with CD, compared to UC.

Recently, there has been an increase in the numbers of studies assessing factors associated with stress which could affect IBD. Graff et al., (2009) evaluated the psychological function and health perception of IBD patients and compared them to a non-IBD population. Overall this study reported that people suffering with IBD had poorer psychological health compared to non-IBD subjects. That study concluded that patients with active IBD frequently used stress-coping strategies which included avoiding

contact with other people, eating more/less and blamed self more than non-IBD individuals (Graff et al., 2009). In addition, certain stressors were more frequently associated with flare ups IBD. A recent survey reported that diarrhoea, one of the most common symptoms of IBD, was correlated with financial, work and school related stressors (Singh et al., 2011). However, it is worth noting that stress in humans is a very complex phenomenon and not only depends on perception, but may involve physiological events as well. In other words, although it is known that hormones like Epinephrine, Norepinephrine and cortisol may have a regulatory effect on human stress (Dettmer et al., 2011; Moore et al., 2011), it is not known whether these hormone levels are influenced by environmental factors, including extremes in temperature, and intensity of sun-light exposure, as levels have not been monitored in IBD patients.

Immune deficiencies in the human host have been connected with IBD, particularly in the case of CD. One of the factors is a deficiency of the *nod2/card15* gene that encodes for a protein which is involved in bacterial recognition. The gene is located on chromosome 16 and contains a frameshift mutation found in CD patients (Ogura et al., 2001). The *nod2* mutation consists of a cytosine insertion at nucleotide 3020 of the gene, and the frequency of this mutation was significantly higher in CD patients (8.2%) compared with control cases (4%) (Ogura et al., 2001). More recently, Bonen et al., (2003) reported three variants of the *nod2* gene, LI007fsinsC, R702W and G908R, and demonstrated an impaired response to bacterial lipopolysaccharide and peptidoglycan which may lead to a reduced ability to activate the regulatory protein nuclear factor-kB (NF-KB).

Currently, it has been reported that autophagy, a process by which eukaryotic cells degrade internally damaged organelles, proteins and internalized pathogens, is associated with CD due to a mutation in the autophagy-related gene *ATG16L1* (autophagy-related 16 like 1) (reviewed by Caprilli et al., 2010). ATG16L1 proteins are involved in the early stages of the autophagy process, working together with NOD2 to eradicate intracellular bacteria. The precise mechanism of how this eradication process works is not clear, but studies have demonstrated that both ATG16L1 and NOD2 are necessary for efficient clearing of internalized pathogens. When mutations were found in both genes, pathogens can amplify the disease, especially in CD patients (Caprilli et al., 2010; Sun et al., 2011).

Another correlation observed in human hosts with CD patients, involves a protein called carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) (Barnich et al., 2007). A study conducted in France reported that CEACAM6 expression was significantly higher in the ileal portion of CD patients compared with controls. However, its expression remained unchanged in the colonic mucosa of both CD and control individuals. Moreover, the study found that a cytokine, interferon gamma (IFN-y), mediated by the presence of pathogenic *E. coli,* strongly stimulated the expression of CEACAM6. It was also indicated that CEACAM6 acted as a receptor for a specific strain of *E. coli,* termed AIEC (Barnich et al., 2007). More recently, Carvalho et al., (2009) overexpressed CEACAM6 in transgenic mice challenged with the AIEC strain of *E. coli* (LF82). They reported that challenged mice had reduced survival rates, increased rectal bleeding, mucosal inflammation and increased proinflammatory cytokine expression.

Both studies strongly correlated CD susceptibility with pathogenic *E. coli,* concluding that this pathogen could take advantage of CD patients starting a cascade of events leading to the disease (Barnich et al., 2007; Carvalho et al., 2009).

6.1.1 Microbial pathogens that influence intestinal inflammation: the role of adherent and invasive *E. coli* (AIEC).

Recent reports indicate that changes in the microbial population of IBD patients, compared with their controls, is known as dysbiosis. However, consensus regarding the precise nature of dysbiosis is inconclusive due to contradictory results. In some instances, a reduction in numbers of Firmicutes was reported, whereas others reported increased numbers of Firmicutes (reviewed by Sartor, 2008; Sun et al., 2011). Moreover, there are unanswered questions of whether microbial population changes are the cause or the consequence of IBD (Sartor, 2008). Despite those differences, most scientists agree that the numbers of recovered *E. coli* from IBD patients is higher compared with non-IBD (control) people based on independent studies (Baumgart et al., 2007; Darfeuille-Michaud et al., 2004; Martin et al., 2004; Kotlowski et al., 2007).

More than 50% of mucosa-associated *E. coli* strains were more frequently isolated from CD compared to the control patients (Martin et al., 2004). Similarly, a study in France reported a higher proportion of *E. coli* in the ileal mucosa of CD patients (36%) versus the controls (6%) (Darfeuille-Michaud et al., 2004). In Canada, utilizing a population-based case-controlled study, it was found that *E. coli* numbers in CD patients were isolated at 3-log units higher than the control (Kotlowski et al., 2007). A culture independent study in the USA, reported a higher number of *E. coli* from the ileum of CD

patients. They also indicated that those *E. coli* strains belonged to a novel phylogenetic group (Baumgart et al., 2007).

The first notion that the *E. coli* strains from CD patients belong to a new pathotype group originated in initial studies conducted in France (Darfeuille-Michaud, 2002). Darfeuille-Michaud demonstrated that the *E. coli* isolates had special characteristics, such that it could not only adhere and invade epithelial cells, but could also survive and replicate within macrophages. They named this strain adherent-invasive *Escherichia coli* (AIEC) (Darfeuille-Michaud, 2002; Glasser et al., 2001). They also noted that this type of *E. coli* had genetic similarities to uropathogenic *E. coli* and avian pathogenic *E. coli*. This idea has been confirmed with investigations using in-silico methods like phylogenetic reconstructions and multi locus sequence typing (MLST) analysis (Baumgart et al., 2007; Sepehri et al., 2009).

One of the hallmarks of AIEC is its ability to invade the epithelium of CD patients. There are a large number of investigations that describe how the invasion process may work. Initially, AIEC adheres to the epithelial surface with the aid of flagella (Claret et al., 2007). The bacterium then anchors itself to the surface of the epithelium using adhesion factors such as FimH (Boudeau et al., 2001; Glasser et al., 2001). FimH is capable of recognizing CEACAM6 in a susceptible host (Barnich et al., 2007). The process of internalization of the bacterium into the epithelium is not totally clear, but important clues indicate that in CD patients, AIEC enters the cells and replicates due to deficiencies in the autophagy and NOD2 pathway (Caprilli et al., 2010). Presently, there are no studies that indicate what genes are activated in AIEC once it is internalized.

However, it is known that the host is still able to respond to the bacterial attack through production of several cytokines, including TNF-α, IFN-γ and interleukins (IL) (Glasser et al., 2001; Barnich et al., 2007; Carvalho et al., 2009).

It is also important to note that the process by which AIEC anchors to epithelial tissues is dependent on several factors, not exclusive to the FimH adhesin. This idea is supported by other studies which found that AIEC uses different pathways to adhere and invade, which include the porin system (OmpC, OmpA), independent of FimH (Rolhion et al., 2007), and a new operon of genes identified as long polar fimbriae (*lpf*) (Chassaing et al., 2011). Rolhion et al., (2010) have reported that a stress response protein (Gp96) is strongly expressed in the epithelial cells of CD patients, and that AIEC can recognize this protein via the outer membrane protein OmpA which consequently attaches and invades the epithelium. Chassaing et al., (2011), discovered that AIEC can target the Peyer's patches in CD patients using the *lpf* operon. Interestingly *lpf* was similarly found in the O157:H7 EDL933 completed genome, but it is not present in other AIEC strains like UM146 as the latter strain is more related to the UPEC CFT073 strain (Krause et al., 2011), which does not have the *lfp* operon (Chassaing et al., 2011).

HYPOTHESES

1. Different culture conditions will affect biofilm formation in environmental *Escherichia coli* regardless of their source.
2. Adhesion and quorum-sensing related genes, particularly AI-2, will have an impact on biofilm formation by wild-type *E. coli* strains.
3. The AI-2 quorum-sensing system will not regulate the flagella-motility factors and biofilm formation in pathogenic *E. coli* AIEC. Instead, AI-3/Epi/Ne may be involved in both situations.
4. The two component system operon *qseBC* will play a direct role in global gene expression during AIEC biofilm formation and will have an impact on the AIEC invasion of eukaryotic cell lines.

OBJECTIVES

Three series of experiments were conducted in *E. coli* in relation to their biofilm forming ability to determine the following:

1. If the addition of CRF and PWF as natural components of *E. coli* growth conditions can affect biofilm formation compared with standard culture medium conditions.
2. To correlate the absence or presence of genes encoding adhesin factors with the AI-2 quorum sensing system and its impact on the biofilm forming ability of environmental *E. coli* strains.
3. To elucidate which system, AI-2 or AI-3/Epi/Ne, has the most important regulatory effect on biofilm formation of pathogenic *E. coli* AIEC.
4. To evaluate whether deleting the two component system operon *qseBC* influences the biofilm forming ability of AIEC.
5. To determine the effect of deleting several essential genes (*luxS, fimH, qseC, qseB*) on the ability of AIEC to invade an epithelial cell line.

Manuscript I

In vitro biofilm formation in environmental strains of *Escherichia coli*: a survey of adhesins and autoinducer-2 (AI-2) quorum sensing genes.

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ABSTRACT

A view is emerging that *E. coli* K-12, the most studied strain in biofilm biology, is not representative of a wider range of environmental *E. coli*. Several factors seem to be associated with biofilm formation, including the presence of adherence factors and the autoinducer-2 (AI-2) QS system. However, this information is based on human intra- and extra-intestinal pathogenic strains of *E. coli* and may not necessarily apply to all non-pathogenic and pathogenic *E. coli*. We evaluated whether the presence of adhesin genes (*aidA, ag43, afa, bma, pap, sfa*), AI-2 signal-related genes (*luxS, lsrA, lsrK, mqsR, ydgG, yceP and yliH*) and media conditions were associated with biofilm formation in a range of *E. coli* strains. A total of 108 non-domesticated strains were screened: 34 human IBD isolates, 32 cattle commensals, 13 soil commensals, 12 pig pathogens and 17 colicin-producing strains. Biofilm formation was evaluated using minimal medium in polyvinyl chloride 96-well plates. To simulate a gut environment, 10% v/v filtered clarified rumen fluid or porcine fecal water was added to the minimal medium. Biofilm formation was dependent on medium conditions ($P<0.01$), but independent of whether the strains were commensal or pathogenic. The presence or absence of adherence and AI-2 signal genes apparently does not explain the ability of wild type *E. coli* to form biofilm. Biofilm formation in wild type *E. coli* strains is highly complex and is dependent on environmental growth conditions.

Key words: biofilm, *E. coli*, clarified rumen fluid (CRF), pig water feces (PWF), quorum sensing

INTRODUCTION

Biofilms are microbial communities that adhere and reproduce while attached to abiotic or biotic surfaces, and are often covered by polymeric substances (Davey and O'toole, 2000; Wood and Bentley, 2007). Living in a biofilm gives microorganisms a competitive advantage, because it protects against shear forces, low pH environments (e.g. gastric stomach), allows for slower growth rates, and filters out toxic compounds. A biofilm phenotype may also be considered a virulence factor. Pathogens in the gastrointestinal tract for example, must usually be in contact with the enterocyte to be infectious (Davey and O'Toole, 2000; Wood and Bentley, 2007).

Biofilm formation has been extensively studied in *Escherichia coli* K-12 and O157:H7 (Wood and Bentley, 2007). It is hypothesized that under low nutrient conditions (Yang et al., 2006; Dewanti and Wong, 1995) quorum sensing (QS) genes are induced (Wood and Bentley, 2007), which potentially regulates expression of adherence genes (Ulett et al., 2007; Ong et al., 2008). In low nutrient environments, microbial cells may enter the biofilm state so that they can survive under nutrient deprivation, a state different from planktonic growth. The QS molecules are present extracellularly, and when these molecules reach a critical level in nutrient limiting conditions, a signal cascade is activated that results in biofilm formation (Hardie and Heurlier, 2008).

The major QS systems described for *E. coli* include the norephinephrine/epinephrine-autoinducer-3 (AI-3) system and the autoinducer-2 (AI-2) system (Wood and Bentley, 2007). AI-2 is a metabolic product of the activated methyl cycle in which sulphate, or cysteine, are converted to homoserine. The *E. coli* gene

product of *luxS* forms 4,5-dihydroxy-2,3-pentanedione, which is highly unstable and rapidly cyclizes to 4-hydroxy-5-methyl-3(2H) furanone, (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran, and furanosyl borate diester. These three molecules are collectively referred to as AI-2 (Hardie and Heurlier, 2008).

In *E. coli* K-12, a number of genes have been associated with the AI-2 system. As discussed above, *luxS* produces AI-2 molecules under low nutrient conditions that are exported and signal other cells to form a biofilm. Consequently, genes for import (*lsrA*), export (*ydgG*), and phosphorylation (*lsrK*) have been identified in *E. coli* K-12 (Xavier and Bassler, 2005; Herzberg et al., 2006; Domka et al., 2006). A number of putative regulatory proteins have also been identified (MqsR, YceP, YliH) (Gonzalez-Barrios et al., 2006). In addition to QS molecules, it can be hypothesized that microbial cell surface structures are involved in the formation of biofilms, because biofilms are always associated the surfaces (Ong et al., 2008; Pratt and Kolter, 1998; Sherlock et al., 2004).

In two recent studies, Reisner et al., (2006), and Skyberg et al., (2007) evaluated a large collection of *E. coli* to determine their ability to form biofilms under different nutrient conditions. Reisner et al., (2006) evaluated both pathogenic and non-pathogenic *E. coli* from human sources. Skyberg et al., (2007) evaluated primarily pathogenic *E. coli* from avian sources. In both studies there was significant variation among *E. coli* strains in their ability to form biofilms. Neither study tried to relate the presence of QS genes or adherence factors to biofilm formation. In this study, biofilm formation in 108 strains of *E. coli* from human, rumen, swine, and soil sources will be evaluated. The frequency of genes associated with AI-2 biofilm formation will be determined.

MATERIALS AND METHODS

Bacterial strains

A total of one hundred and eight *E. coli* isolates were obtained from various gut samples which were available in our laboratory. Thirty-four isolates were obtained from humans with inflammatory bowel disease (Kotlowski et al., 2007), 32 from cattle feces (Walkty, 2007), 13 from hog manure spread on pasture (Walkty, 2007), 12 were clinical *E. coli* K88+ (Dr. Carlton Gyles, University of Guelph), and 17 produced inhibitory compounds to *E. coli* K88 and were isolated from various gut sources (Setia, 2007). All strains were stored at -80°C in a 1:1 mixture of Luria-Bertani (LB) broth and 30% glycerol solution.

Growth conditions

Bacteria were maintained on LB medium. For static biofilm assays three media types were used. Minimal medium (M9) contained per liter: 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 1 mmol of $MgSO_4$, 4 g glucose, and 4 g casamino acids (Poritsanos, 2006). Clarified rumen fluid medium (CRF) contained M9 plus 10% clarified rumen fluid (CRF). The CRF was obtained from a fistulated steer consuming alfalfa based diet. Rumen fluid were filtered through four layers of cheese cloth and incubated at 39°C for 16 hours so that residual soluble sugars would be consumed by the microorganisms in the rumen fluid. Thereafter, the rumen fluid was centrifuged at 10,000 rpm for 30 min. The supernatant was decanted into falcon tubes without disturbing the microbial pellet and

stored at -20°C. Thawed CRF was added to sterile M9 through a 0.22 μM filter (Millipore). Pig fecal water medium (PFW) was M9 plus 10% pig fecal water (PFW). The PFW was obtained from healthy adult pigs fed an antibiotic-free diet containing soybean meal, wheat, and barley. Feces samples were collected from pens, resuspended in two volumes of autoclaved distilled water. The suspension was the processed as for CRF.

Static biofilm assay.

E. coli strains were grown overnight in LB medium at 37°C until they reached an optical density (OD_{600}) of between 0.8 and 1. Stationary cultures were added to test medium in a ratio of 1:100. A total of 200 μl of cell suspension was added to 96 well polyvinylchloride microtiter plates (Becton Dickinson Labware). Negative control wells contained medium only, and each strain was tested six times per assay. Plates were inoculated aerobically without shaking at 37°C for 48 h. The cells were stained by the addition of 25 μl of a 1% crystal violet (CV) solution and allowed to incubate at room temperature for 15 minutes. Plates were rinsed several times with H_2O to remove any unbound cells. Biofilm growth was detached by adding 200 μl of 95% ethanol, and the suspension was then transferred to a new plate. The biofilm was then quantified by measuring the OD at 595 nm in a MicroPlate Reader (BIORAD, California US). Readings were corrected using the negative control (Poritsanos, 2006). All experiments were carried out in two independent experiments and thus obtaining 12 readings per strain per medium type.

Bacterial biofilms were classified based on a OD cut-off ODc as described by Stepanovic et al., (2004). The ODc was defined as three standard deviations from the OD mean of the negative control. No biofilm formation was OD < ODc; a weak biofilm former was ODc < OD < (2 X ODc); a moderate was biofilm former (2 X ODc) < OD < (4 X ODc); and, a strong biofilm former was (4 X ODc) < OD (Stepanovic et al., 2004).

PCR conditions

DNA extraction was performed according to Sambrook and Russell, (2001). All the PCR reactions were carried out in a Techne TC-512 thermal cycler (Cambridge Ltd, Duxford UK). Each 20 µl PCR reaction contained 2 µl PCR buffer (20 mmol l-1 Tris-HCL, pH 8.4; 50 mmol l-1 KCl), 0.2 µl $MgCl_2$ (1.5 mmol l-1), 0.5 µl; dNTPs (0.5 mmol l-1; FisherScientific, USA), 0.5 µl of each primer (25 pmol), 0.5 unit Taq DNA polymerase (Lucigen, Corporation Middleton, USA) and approximately 15 ng of genomic DNA. The amplification conditions were one cycle of denaturation at 94°C for one minute, followed by 36 cycles of denaturation at 94°C for one minute, annealing at 60°C for one minute, and extension at 72°C for one minute. The final cycle had an extension temperature of 72°C for two minutes. The PCR products were separated by electrophoresis in 2% agarose in order to determine the presence or absence of the genes. Conditions were optimazed so that the same annealing temperature for all primer pairs could be used (Table 1).

Table 1. Primers used in manuscript I

***E coli* phylogenetic groups**	**Annealing ^{o}C**	**Source**
ChuAf 5' GGACGAACCAACGGTCAGGAT	60	Clermont *et a*l 2000
ChuAr 5' GCCGCCAGTACCAAAGACACG	60	Clermont *et a*l 2000
Yjaf 5' CGTGAAGTGTCAGGAGACGCTGC	60	Clermont *et a*l 2000
Yjar 5' GCGTTCCTCAACCTGTGACAAACC	60	Clermont *et a*l 2000
Tsp1 5' GGGAGTAATGTCGGGGCATTCAG	60	Clermont *et a*l 2000
Tsp2 5' CATCGCGCCAACAAAGTATTACGCAG	60	Clermont *et a*l 2000
***E coli* adhesins**		
AIDA1 5' TATGCCACCTGGTATGCCGATGAC	60	Kotlowski *et al* 2007
AIDA2 5' ACGCCCACATTCCCCCAGAC	60	Kotlowski *et al* 2007
Ag43F 5'TGACACAGGCAATGGACTATGACCG	60	Kotlowski *et al* 2007
Ag43R 5'GGCATCATCCCGGACCGTGC	60	Kotlowski *et al* 2007
Afaf 5' TATGGTGAGTTGGCGGGGATGTACAGTTACA	60	Kotlowski *et al* 2007
Afar 5' CCGGGAAAGTTGTCGGATCCAGTGT	60	Kotlowski *et al* 2007
AggRf 5' GAGTTAGGTCACTCTAACGCAGAGTTG	60	Kotlowski *et al* 2007
AggRr 5' GACCAATTCGGACAACTGCAAGCATCTAC	60	Kotlowski *et al* 2007
BmaEf 5' CTAACTTGCCATGCTGTGACAGTA	60	Kotlowski *et al* 2007
BmaEr 5' TTATCCCCTGCGTAGTTGTGAATC	60	Kotlowski *et al* 2007
Sfaf 5' CGGAGGAGTAATTACAAACCTGGCA	60	Kotlowski *et al* 2007
Sfar 5' CTCCGGAGAACTGGGTGCATCTTAC	60	Kotlowski *et al* 2007
PapF 5' CCGGCGTTCAGGCTGTAGCTG	60	Kotlowski *et al* 2007
PapR 5' GCTACAGTGGCAGTATGAGTAATGACCGTTA	60	Kotlowski *et al* 2007
***E. coli* AI-2 quorum sensing genes**		
lsrA (F) 5’GATCTGCATAGTCTGGCAGGATCG	60	This study
lsrA(R) 5’CGTCTCGGCCAGTTCTGTGC	60	This study
lsrK (F) 5’ GCGCCAATGTGGATGCCAGAG	60	This study
lsrK (R) 5’ CAGACCGCAGAGTTCCGCC	60	This study
luxS (F) 5’GCGCTTCTGCGTGCCGAAC	60	This study
luxS (R) 5’GGTAAGTGCCACACTGGTAGACG	60	This study
MqsR(F)5’CGAGACGATCAGTACGTCATGAATTAC	60	This study
MqsR(R)5’CGCACACCACATACACGTTTGAGTC	60	This study
yceP (F)5’GGTGGATGGTCATGTCATGTTAATGAC	60	This study
yceP (R)5’CATTCAGACTCATCCGCTCGTAG	60	This study
ydgG (F) 5’GAGTTGACGCGGACGTTACCGC	60	This study
ydgG (R) 5’CCGCCAGCACCAGCAACGC	60	This study
yliH (F) 5’GACAGACAGCGAATCGATCTGCTG	60	This study
yliH (R) 5’CTCAAGCATGGCGTGCTTCTGC	60	This study

Statistical analysis.

Data obtained from static biofilm assays were log transformed to stabilize the variance and to make an approximation to the normal distribution. Data were subjected to ANOVA using the REML PROC MIXED procedures of SAS version 9.0 (SAS Inst. Inc., Cary, NC). The variables origin and phylogeny were merged into an origin+phylogeny variable, due to the fact that the phylogenetic typing was not complete in all the groups. The effects of origin+phylogeny, media and their interactions were considered fixed. Trial, strains and their interactions were considered random. Multivariance analysis was conducted in order to determine the effect of adhesins and AI-2 QS genes on biofilm formation. The presence and absence of these genes was compared with biofilm data using a canonical correspondence analysis in CANOCO software (Ter Braak and Smilauer, 2002).

RESULTS

In-vitro biofilm assay conditions

A total of 108 diverse *E. coli* strains from different origins were analyzed for biofilm formation included in this study. All the strains were well characterized from previous studies conducted at the Department of Animal Science, University of Manitoba.

Initially all the strains were grown in LB medium plus glucose (0.4%) before starting the biofilm assay. This standardization was adhered to because many wild type strains were unable to reach the desirable OD (0.8-1) using minimal media. Biofilm formation in the wild-type *E. coli* was tested using three different media conditions. Minimal media (M9) supplemented with glucose (0.4%) and casamino acids (0.4%) served as standard laboratory media. To simulate the gut conditions, M9 was supplemented with a 10% v/v quantity of clarified rumen fluid (CRF) and porcine water feces extract (PWF).

Biofilm formation varies within environmental *E. coli*.

Table 2. ANOVA test of biofilm formation according to OrigenPhylogeny, Media and Strain *

Fixed factors	**F- Value**	***P*-Value**
OrigenPhylogeny	3.18	<.0001
Media	28.67	<.0001
OrigenPhylogeny x Media	1.29	0.1340
Random factors	**Z- Value**	***P*-Value**
Trial	0.34	0.3553
Strain	4.89	<.0001

(*) The results suggest that factors OrigenPhylogeny, Media and Strain are significant at the 99% confidence level with the exception of Trial. Moreover, the two level interactions factor effect was not statistically significant.

For the *in-vitro* biofilm formation experiments M9 was used as standard laboratory medium whereby the majority of isolates exhibited a high ability to form biofilm (Tables 3 and 4). Human *E. coli* strains had a high number of biofilm formers (18 moderate), 13 were classified as weak, and three strains were non-biofilm formers (Table 3). Cattle strains had also a high concentration of biofilm formers (25 moderate), where only six and one were categorized into weak and non biofilm formers, respectively (Table 3). A similar trend was found in *E. coli* of soil, pigs K88+ and colicin sources with 11, 11 and 13 strains being classified into moderate biofilm formers, respectively (Table 4).

When CRF and PWF were included as supplements to the M9 medium, biofilm former strains were lower than when compared to M9 alone. Human strains showed a dramatically decreased number of biofilm former strains under CRF supplemented conditions, with 21 and 12 strains classified into weak and non-biofilm formers,

respectively. The majority of strains supplemented with PWF (31) became weak biofilm formers (Table 3). Conversely, cattle strains were prone the weak class (24) using CRF. The biofilm categorization was more distributed when PWF was used, with 12 and 20 strains belonging to the weak and moderate classes, respectively (Table 3). A similar situation occurred with the rest of the strains in soil, pigs, and colicin groups when CRF and PWF were used. The soil group had the majority of strains (12) clustered into the weak biofilm class in CRF, whereas in PWF the proportion was seven and five strains grouped into the weak and moderate biofilm class respectively (Table 4). Pig strains were similarly classified into the weak and moderate class for CRF and PWF with four and eight, plus two and ten total strains respectively (Table 4). Finally, colicin-producing strains had a well distributed number of strains when CRF was used, with nine and six strains in the weak and moderate classes respectively; while most of the strains (12) were clustered into the weak biofilm category using PWF (Table 4).

From this scenario it can be seen that biofilm formation was better when using a standard laboratory medium (M9). Results of this research agree with previous studies conducted by Reisner et al. (2006) where it was found that environmental *E. coli* strains from humans formed less biofilm when complex media conditions were used. Nevertheless, our results differ from the study by Skyberg et al. (2007) which reported a high number of avian *E. coli* being categorized as non-biofilm formers, when the biofilm experiment conditions were conducted using standard rich lab media such as BHI and TSB.

Table 3. Biofilm formation in *E. coli* from human and cattle sources according to phylogenetic groups and media conditions.

	Non B.F	Weak	Moderate
	Human *E. coli* π		
M. media + 0.4% glucose/0.4% casamino acids			
A	1	2	2
B1	1	1	3
B2	1	10	10
D	0	0	3
Total	**3**	**13**	**18**
M.m + 10% CRF*			
A	2	3	0
B1	2	3	0
B2	6	14	1
D	2	1	0
Total	**12**	**21**	**1**
M.m + 10% PWF**			
A	0	5	0
B1	0	5	0
B2	3	18	0
D	0	3	0
Total	**3**	**31**	**0**
	Cattle *E. coli* ξ		
M. media + 0.4% glucose/0.4% casamino acids			
A	0	2	4
B1	1	3	18
B2	0	1	3
Total	**1**	**6**	**25**
M.m + 10% CRF			
A	0	5	1
B1	0	16	6
B2	0	3	1
Total	**0**	**24**	**8**
M.m + 10% PWF			
A	0	0	6
B1	0	11	11
B2	0	1	3
Total	**0**	**12**	**20**

(π) = strains obtained from diseased patients with ulcerative colitis and Crohn's disease; (ξ) = strains obtained from steers treated or non-treated with hog manure; (*) = clarified rumen fluid; (**) = porcine water feces extract.

Table 4. Biofilm formation in *E. coli* from soil, pig and colicin sources according to phylogenetic groups and media conditions.

	Non B.F	Weak	Moderate
	Soil *E. coli* $^{\gamma}$		
M. media + 0.4% glucose/0.4% casamino acids			
A	0	1	7
B1	1	0	3
D	0	0	1
Total	**1**	**1**	**11**
M.m + 10% CRF*			
A	0	8	0
B1	0	3	1
D	0	1	0
Total	**0**	**12**	**1**
M.m + 10% PWF**			
A	0	4	4
B1	1	2	1
D	0	1	0
Total	**1**	**7**	**5**
	Pig K88^{+} *E. coli* $^{\delta}$		
M. media + 0.4% glucose/0.4% casamino acids			
A	1	0	10
B1	0	0	1
Total	**1**	**0**	**11**
M.m + 10% CRF			
A	0	4	7
B1	0	0	1
Total	**0**	**4**	**8**
M.m + 10% PWF			
A	0	2	9
B1	0	0	1
Total	**0**	**2**	**10**
	Colicin *E. coli* $^{\lambda}$		
M. media + 0.4% glucose/0.4% casamino acids			
A	0	0	4
B1	0	4	9
Total	**0**	**4**	**13**
M.m + 10% CRF			
A	0	1	3
B1	2	8	3
Total	**2**	**9**	**6**
M.m + 10% PWF			
A	0	2	2
B1	2	10	1
Total	**2**	**12**	**3**

(γ) = strains obtained from a field before spreading hog manure; (δ) = strains were a gift from Dr C.L Gyles University of Guelph, Ontario; (λ) = isolates were developed at the Department of Animal Science, University of Manitoba. (*) = clarified rumen fluid; (**) = porcine water feces extract

Biofilm formation according to media condition, phylogenetic type and strains

The log transformed data A595 of all 108 strains was subjected to ANOVA analysis using REML in the SAS software to determine whether the effect of origin, media condition, phylogenetic typing and their interaction factors had an effect over biofilm formation. According to the ANOVA test (Table 2), the variables origin+phylogeny, media and strains were highly significant ($P< 0.01$). In this case, we have merged the variables origin and phylogeny in order to make the SAS program recognize the difference among the groups, due to the fact that the phylogenetic typing was not found in all groups (Tables 3 and 4). Based on ANOVA analysis, the biofilm formation was statistically significant between phylogenetic groups from different origins ($P< 0.01$). The majority of human strains, for example, were clustered into the phylogenetic virulent group B2 (21); in cattle strains the most abundant phylogenetic group was B1 (22); in soil and pig strains the prevalent phylogenetic group was A (eight and 11 respectively), and in colicin-producing *E. coli* bacteria the most abundant was B1 (13) (Tables 3 and 4). Additionally, Tables 3 and 4 demonstrate the variability of the biofilm in these principal phylotypes. In general, it can be seen that media conditions played a strong role in biofilm formation which shifted systematically when growth media conditions changed ($P< 0.01$). These results concur with previous reports (Reisner et al., 2006). Additionally, the multivariance analysis test corroborates that media conditions play a significant role in biofilm formation, indicated by the presence of arrows in the plot pointing out the different media conditions (Figure 1).

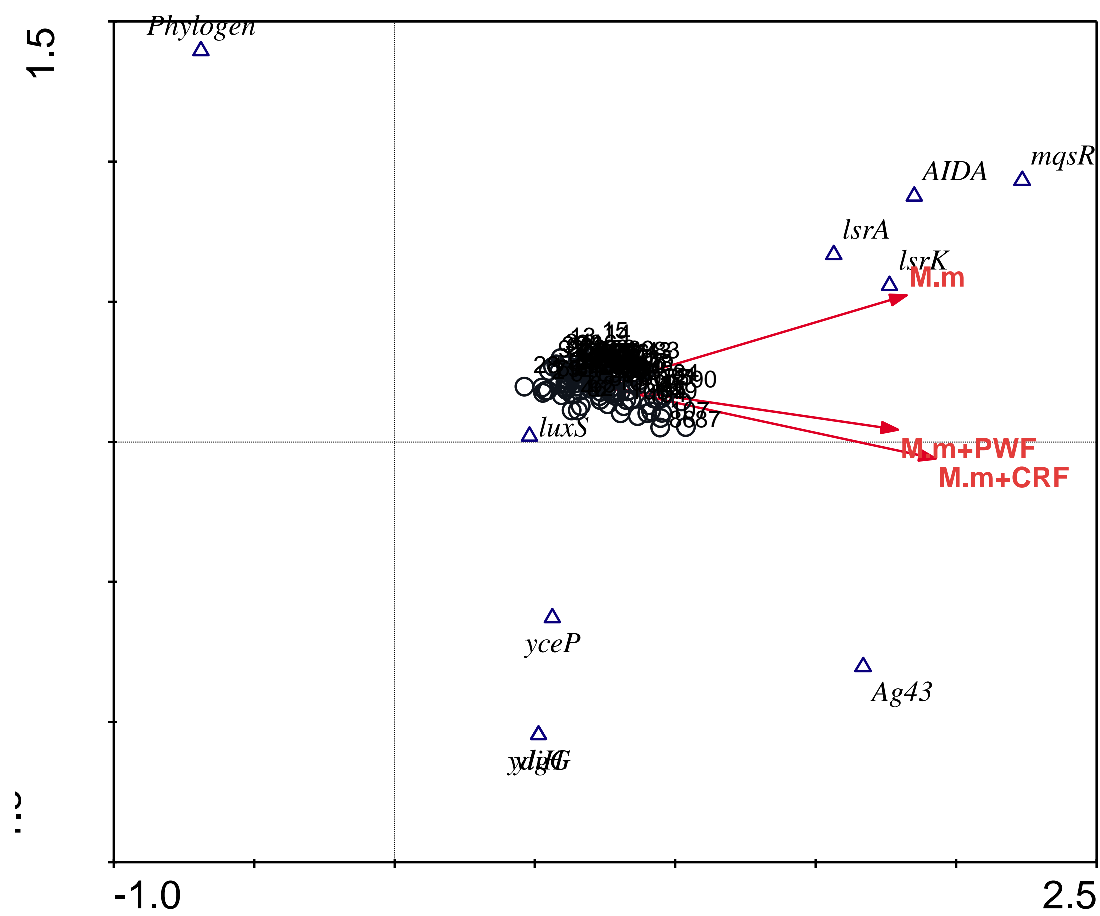

Figure 1. Multivariance analysis of biofilm formation, adhesins and AI-2 quorum sensing genes.

Presence of adhesins, AI-2 quorum sensing genes and biofilm formation

Many investigations have linked the presence of adhesins with virulent *E. coli* strains and biofilm formation. In this study, we have screened all of our strains to detect the presence of several adhesins genes by PCR reactions based on a previous study conducted by Kotlowski et al.,(2007). As outlined in Table 5, our initial screen revealed that only *aidA* and *ag43* were present in almost all of the *E. coli* strains. These genes were used for the multivariance analysis (Figure 1). The multivariance test indicated that the presence of these two adhesins did not have a significant impact on biofilm formation (Figure 1). These results with previously reported studies (Sherlock et al., 2004; Ulett et al., 2007b; Yang et al., 2006) where the role of adhesins AIDA plus Ag43 was evaluated at an earlier stage of biofilm formation (24 hours). In our study, biofilm experiments were conducted at later stages (48 hours), suggesting that this difference in time frame might provide a plausible reason for our present results.

Table 5. Survey of adhesins and phylogeny in wild type *E. coli* strains.

Adhesins	Phylogenetic groups			
	A	B1	B2	D
		Human strains (34)*		
AIDA	4 (1)†	3 (2)	5 (15)	4 (0)
Ag43	3 (2)	2 (3)	13 (7)	1 (3)
Afa	0	2 (3)	4 (16)	0
AggR	0	0	0	0
Bma	0	0	0	0
PAI-1	0	1(4)	5 (15)	0
Sfa	0	0	6 (14)	0
		Cattle strains (32)		
AIDA	6(0)	22 (0)	4 (0)	0
Ag43	6(0)	10 (12)	2 (0)	0
Afa	0	0	0	0
AggR	0	0	0	0
Bma	0	0	0	0
PAI-1	5(1)	3 (19)	2 (2)	0
Sfa	0	0	0	0
		Soil strains (13)		
AIDA	2(6)	3 (1)	0	1 (0)
Ag43	5(3)	2 (2)	0	0
Afa	0	0	0	0
AggR	0	0	0	0
Bma	0	0	0	0
PAI-1	0	0	0	0
Sfa	0	0	0	0
		Pig K88+ strains (12)		
AIDA	11(0)	1 (0)	0	0
Ag43	11(0)	1 (0)	0	0
Afa	0	0	0	0
AggR	0	0	0	0
Bma	0	0	0	0
PAI-1	0	0	0	0
Sfa	0	0	0	0
		Colicin strains (17)		
AIDA	4 (0)	4 (0)	0	0
Ag43	13 (0)	13 (0)	0	0
Afa	0	0	0	0
AggR	0	0	0	0
Bma	0	0	0	0
PAI-1	3(1)	11 (3)	0	0
Sfa	0	0	0	0

(†) = numbers in parenthesis indicate number of strains without the gene. (*) = total number of strains inside the group

Seven genes involved in the autoinducer-2 (AI-2) QS system were surveyed in all strains (Table 6). Based on PCR reactions, it can be observed that all strains harbored the *luxS* gene. In addition, several genes with diverse functions, including transporting AI-2 (*lsrA*), uptake (*lsrK*), exporting AI-2 (*ydgG*) and genes responsible to regulate motility and fimbria by using AI-2 signal (*mqsR, yliH, yceP*), were included in the survey. Surprisingly, many of these genes were absent in several *E. coli* strains. Of particular interest is the motility QS regulator (*mqsR*) gene, which presented a greater number of absences in *E. coli* from human and cattle origin. This gene was present mainly in *E. coli* of phylogenetic group A in which K-12 strains are clustered, thus suggesting the *mqsR* gene may be unique to this bacterium. The multivariance analysis test also found that the absence and presence of AI-2 QS related genes have no statistical significance over biofilm formation, since all the genes were included in the plot, however, there were no arrows linked with them (Figure 1).

Table 6 Autoinducer-2 (AI-2) quorum sensing genes in environmental *E. coli*

	Phylogenetic groups			
AI-2 quorum sensing genes	A	B1	B2	D
	Human strains (34)*			
Synthesis of AI-2 (*luxS*)	5(0)†	5(0)	20(0)	4(0)
Import of AI-2 (*lsrA*)	4(1)	5(0)	5(15)	4(0)
Phosphorylation of AI-2 (*lsrK*)	4(1)	5(0)	4(16)	4(0)
Export of AI-2 (*ydgG*)	4(1)	5(0)	19(1)	4(0)
QS regulator protein (*mqsR*)	4(1)	1(4)	2(18)	1(3)
Hypothetical QS protein (*yceP*)	4(1)	5(0)	17(3)	4(0)
Hypothetical QS protein (*yliH*)	4(1)	5(0)	19(1)	4(0)
	Cattle strains (32)			
Synthesis of AI-2 (*luxS*)	6(0)	22(0)	4(0)	0
Import of AI-2 (*lsrA*)	6(0)	22(0)	4(0)	0
Phosphorylation of AI-2 (*lsrK*)	6(0)	19(3)	4(0)	0
Export of AI-2 (*ydgG*)	6(0)	22(0)	4(0)	0
QS regulator protein (*mqsR*)	2(4)	3(19)	1(3)	0
Hypothetical QS protein (*yceP*)	6(0)	22(0)	4(0)	0
Hypothetical QS protein (*yliH*)	6(0)	22(0)	4(0)	0
	Soil strains (13)			
Synthesis of AI-2 (*luxS*)	8(0)	4(0)	0	1(0)
Import of AI-2 (*lsrA*)	1(7)	3(1)	0	1(0)
Phosphorylation of AI-2 (*lsrK*)	1(7)	3(1)	0	1(0)
Export of AI-2 (*ydgG*)	1(7)	3(1)	0	1(0)
QS regulator protein (*mqsR*)	8(0)	4(0)	0	1(0)
Hypothetical QS protein (*yceP*)	1(7)	3(1)	0	1(0)
Hypothetical QS protein (*yliH*)	1(7)	3(1)	0	1(0)
	Pig strains (12)			
Synthesis of AI-2 (*luxS*)	11(0)	1(0)	0	0
Import of AI-2 (*lsrA*)	11(0)	1(0)	0	0
Phosphorylation of AI-2 (*lsrK*)	11(0)	1(0)	0	0
Export of AI-2 (*ydgG*)	11(0)	1(0)	0	0
QS regulator protein (*mqsR*)	11(0)	1(0)	0	0
Hypothetical QS protein (*yceP*)	11(0)	0(1)	0	0
Hypothetical QS protein (*yliH*)	11(0)	1(0)	0	0
	Colicin strains (17)			
Synthesis of AI-2 (*luxS*)	4(0)	13(0)	0	0
Import of AI-2 (*lsrA*)	4(0)	13(0)	0	0
Phosphorylation of AI-2 (*lsrK*)	4(0)	13(0)	0	0
Export of AI-2 (*ydgG*)	4(0)	13(0)	0	0
QS regulator protein (*mqsR*)	4(0)	10(3)	0	0
Hypothetical QS protein (*yceP*)	4(0)	13(0)	0	0
Hypothetical QS protein (*yliH*)	4(0)	13(0)	0	0

(†) = numbers in parenthesis indicate number of strains without the gene. (*) = number of strains inside the group.

DISCUSSION

One of the most studied biofilm forming bacteria is *E. coli*, but the large majority of these studies have been conducted with the well-characterized laboratory strains K-12 and O157:H7. Consequently it is not known how applicable the information garnered from these strains is correlated with *E. coli* biofilm formation in general. The studies of Reisner et al. (2006) and Skyberg et al. (2007) evaluated a large set of *E. coli* strains and found that the ability to form biofilms varied extensively. Both studies concluded that medium type was an important predictor of biofilm formation, and nutrient-poor formulations were more likely to result in greater biofilm formation. Neither of these studies correlated the presence of genes for QS or adherence, both of which are hypothesized to be important predictors of biofilm formation (Hardie and Heurlier, 2008). Our study also tested strains in three medium types, two of which were designed to simulate the digestive tract environment.

In this study, phylotype defined by the ABD system (Clermont et al., 2000) was used to evaluate all of our isolates (Table 2). Skyberg et al. (2007) evaluated the relationship between 105 avian pathogenic and 103 avian fecal commensal *E. coli* strains and found a significant relationship between ABD phylotype and biofilm forming ability. We performed a multivariance analysis and found no statistically significant relationship between phylotype and biofilm formation. Differences between the two studies may be attributed to differences in the strain examined. Skyberg et al. (2007) used only avian strains while we evaluated a much more diverse collection of strains.

Data from this investigation illustrate the variation in degree of biofilm formation in environmental *E. coli* under in-vitro conditions. Similarl to other studies, it was found that media conditions had a marked impact on biofilms (Reisner et al., 2006; Skyberg et al., 2007; Yang et al., 2006). In general, this report found that minimal media (M9) permitted a greater formation of biofilm compared with M9 supplemented either with CRF or PWF. However, on a few occasions, adding both components into M9 increased the formation of biofilms for some strains. Interestingly this situation can lead to several hypotheses regarding biofilm formation. For instance, there is a lack of knowledge about the composition of CRF and PWF. CRF is known to be a source of volatile fatty acids which are mainly acetic, propionic and butyric acid (Calberry et al., 2003). Additionally, it has been reported that CRF contains acyl homoserine lactone (AHL) signals (Erickson et al., 2002). Despite this information, many elements of CRF and PWF are still unknown, leading to speculation that at certain points *E. coli* might use some of these substances to either increase or decrease biofilm formation.

Autotransporter protein adhesins can also have an impact over biofilm formation (Van Houdt and Michiels, 2005). In particular, the role of AIDA and Ag43 in biofilm formation in pathogenic *E. coli* strains is well documented (Sherlock et al., 2004; Ravi et al., 2007; Ulett et al., 2007b). Ag43 promotes biofilm by enhancing microcolony formation (Van Houdt and Michiels, 2005). AIDA can also enhance the formation of biofilm in pathogenic and nonpathogenic *E. coli* under *in-vitro* conditions (Sherlock et al., 2004; Ravi et al., 2007). In most cases, studies conducted on AIDA and Ag43 have mainly focused on uropathogenic and K12 strains. In our survey we included a broad

non-domesticated *E. coli* strain which varied from those strains. Despite our multivariance analysis indicating that AIDA and Ag43 did not have a significant impact on biofilm formation, it does not necessarily indicate that these adhesins are not involved in biofilm formation. It is probably safe to argue these adhesins played a role in the initial stages of biofilm formation when the bacteria are attaching to the surface.

Surprisingly, our results revealed the majority of wild-type *E. coli* in this survey did not present many genes involved in AI-2 signalling, particularly the *mqsR* gene. It is noteworthy that this gene was present in *E. coli* strains belonging to the phylogenetic group A where K-12 is grouped (Clermont et al., 2000). Interestingly the statistical analysis indicated our strains formed biofilms despite the presence and absence of genes included in this survey. The situation may contrast with investigations of the AI-2 system and biofilm formation in K-12 strains where a mutation in the *luxS* gene impeded the synthesis of AI-2 which can affect the biofilm (Wood and Bentley, 2007). Based on our data, it is not possible to define which system (AI-2 or AI-3) is prevalent for our situation. It can be hypothesized that our wild-type strains are able to use the AI-3 system to regulate their biofilm formation instead. Notably, many strains came from gastrointestinal origins like cattle feces and human gut tissue. Perhaps under gut conditions *E. coli* requires a specific QS system to recognize and colonize inside the host, and such a case could be applicable for the AI-3 system (Hughes and Sperandio, 2008).

CONCLUSION

In summary, it is clear that media conditions profoundly affected biofilm formation by wild-type *E. coli* strains in this study. Biofilm formation by *E. coli* isolates increased when using a standard lab medium (M9), with the exception of a few isolates where adding CRF or PWF further enhanced biofilm formation. Clearly, we still do not have sufficient knowledge of all the components present in both CRF and PWF to know how biofilm formation of wild type *E. coli* occurs in gut systems. Interestingly, we found that common attaching factors such as adhesins do not impact the development of biofilm under the conditions conducted in this investigation. It was also observed that several genes involved in the AI-2 QS system were missing in our wild-type *E. coli*, and more importantly the bacteria were able to form biofilms despite the lack of these genes.

This is the first study that has assessed the biofilm formation ability of a range of isolates, primarily from the gut, but more importantly from humans. Further studies are needed to address the fact that several adhesins and the AI-2 QS related genes were missing, particularly in the human *E. coli* strains (Tables 5 and 6), and that those strains were capable of forming biofilms in spite of observed absence of those genes. Thus, we hypothesized that it may well be possible that other QS systems are at play. In Manuscript II a human pathogenic *E. coli* (AIEC UM146) strain, isolated from the ileal portion of a Crohn's disease patient (Krause et al., 2011). This strain was chosen because it must adhere to the epithelium, but does not have a LEE, before it can invade. Thus it was an ideal strain to use because several AI-2 genes were missing but biofilm formation may be a key part of its virulence strategy.

Manuscript II

Biofilm formation in adherent invasive *Escherichia coli* is conditionally regulated by bacterial adrenergic receptors

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ABSTRACT

Adherent invasive *Escherichia coli* (AIEC) are associated with inflammatory bowel disease (IBD) patients and particularly with Crohn's Disease (CD). AIEC can adhere and invade epithelial cells and macrophages via carcinoembryonic antigen-related cell adhesion molecule 6, and individuals that have autophagy pathway risk genes may be most susceptible. Stress and/or depression precede flaring reactions; the former is usually associated with the release of catecholamines like epinephrine (Epi) and norepinephrine (Ne). Specific microbial receptors to Epi and NE have been identified in *E. coli* O157:H7 and induce biofilm formation. The ability of AIEC to increase biofilm formation in an Epi dependent manner may help explain inflammatory flares observed in CD patients. We hypothesized that biofilm formation by AIEC is dependent on Epi and the Presence of the hypothesized bacterial Epi receptor, *qseC*. A well-characterized AIEC strain (UM146) was grown in a static biofilm assay. Genes associated with biofilm formation (*lux*S, *fim*H, *qse*B, *qseC*) were mutated with the lambda red system. Biofilm formation was Epi dose-dependent and the greatest effect was observed at 12 hours. Bacterial motility, a key physiological factor in biofilm formation, was not affected by the *lux*S or *fim*H mutants but was significantly reduced by *qseC*. We conclude that AIEC increased motility in an Epi dose-dependent fashion is conditional on the presence of Epi receptor, *qseC*. This is the first study which directly implicates a specific member of the gut microbiome with inflammatory flares observed in IBD patients.

Key words: AIEC *E. coli*, biofilm, epinephrine, RT-PCR

INTRODUCTION

It is accepted that inflammatory bowel disease (IBD) lies at the intersection of a genetically susceptible host, a dysfunctional intestinal immune response, and one or more environmental triggers (Parkey and Sartor, 2009). A great deal of effort has been expended in trying to understand the role of the microbiome in respect to an environmental trigger in IBD. Substantial advances have been made in elucidating the nature of the microbial dysbiosis that occurs in patients (Parkey and Sartor, 2009). One of the most important advances has been the recognition that an adherent and invasive *E. coli* (AIEC) has a potentially important function in the development of the disease (Kotlowski et al., 2007; Parkey and Sartor, 2009; Rolhion and Darfeuille-Michaud, 2007). AIEC, which has been shown to be associated with 25 to 35% of IBD patients, is found in granulomas and selectively binds to carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) (Carvalho et al., 2009).

A second, and very intriguing finding, is that stress may serve as an environmental trigger in disease exacerbation in IBD (Collins and Bercik, 2009). It has been recently discovered that epinephrine (Epi) influences the gut microbiome (Kendall and Sperandio, 2007). Studies with a rodent model of depression have been the first to directly demonstrate that depression precedes colitis (Ghia et al., 2008). Hence, there is evidence that stress and depression may have an important role in IBD. When an individual experiences stress, corticotrophin releasing hormones (CRH) are released from the brain and act on the adrenal gland (Taché et al., 2009). Two hormones released in response to

CRH are norepinephrine (Ne) and epinephrine (Epi), both having a direct influence on the gut epithelial tissue. Epi has more recently been found to have a direct influence on the gut microbiome by stimulating the formation of microbial biofilms adhering to the epithelial tissue (Kendall and Sperandio, 2007).

Biofilm formation has medical importance since many bacteria species are capable of resisting antibiotic treatments and host immunological defences (Boudeau et al., 2001). The majority of biofilm studies in *E. coli* have been conducted principally on K-12 (Pratt and Kolter, 1998) and uropathogenic strains (Schembri and Klemm, 2001). Extensive investigations have described the complex physiology of biofilm formation in *E. coli* (reviewed by Beloin et al., 2008). One important aspect of biofilm physiology deals with the synthesis of flagellar factors and type 1 pili, both of which are implicated in the bacterium dispersion and adherence to abiotic and biotic surfaces (Goller and Romeo, 2008).

Several biofilm-related genes were regulated in *E. coli* via quorum sensing (QS), including the master flagellar operon *flhDC* which controls the *E. coli* swimming ability (Wood and Bentley, 2007). Two QS systems have been implicated in the motility regulatory pathway in K-12 and O157:H7 strains: autoinducer 2 (AI-2) and autoinducer 3-epinephrine-norepinephrine (AI-3/Epi/Ne) respectively (Clarke et al., 2006; Hughes et al., 2009; Wood and Bentley, 2007). In the K-12 strain, AI-2 plays a role in bacterial motility and hence affects biofilm formation using the two-component system operon, *qseBC*, and the flagellar master regulon *flhDC*. Both are mediated by a motility QS regulator gene (*mqsR*) (González-Barrios et al., 2006). Conversely, in pathogenic *E. coli*

O157:H7 a metabolic role for the *luxS* gene has established through detoxifying the cell from the S-adenosyl-methionine (SAM) compound (Clarke and Sperandio, 2006; Walters and Sperandio, 2006). Instead, O157:H7 flagella and motility expression is regulated by the AI-3/Epi/Ne system via the two-component operon *qseBC* (Sperandio et al., 2002). The process starts with the cognate receptor (QseC) that senses the three molecules Epi, Ne and AI-3 allowing it for autophosphorylation and transfer of the phosphate group to the RR QseB. Once QseB is activated it regulates the flagellar regulon, *flhDC*, which will control the flagella-motility network (Clarke et al., 2006).

In this study we investigated the role of Epi in biofilm formation and motility of the well-characterized AIEC strain UM146 (Krause et al., 2011). Our results indicate that the AIEC may use the AI-3/Epi/Ne QS system and not the AI-2 system. We also demonstrate that QseC is the receptor for Epi in AIEC that controls genes involved in the flagella-motility network. However, the AIEC biofilm formation process may be interconnected by several pathways that regulate this complex process. This is the first study that connects a stress hormone (Epi) with a pathogenic *E. coli* strain from IBD sources.

MATERIALS AND METHODS

Strains and culture conditions

E. coli UM146 was chosen to perform biofilm and deletion mutation experiments due to its ability to rapidly colonize and invade cell lines (Krause, unpublished results). The *E. coli* K-12 and O157:H7 strains were used as controls for PCR reactions. The strains were grown in Luria-Bertani (LB) broth medium at 37°C and stock cultures were maintained in glycerol (30%) at -80°C until further use. Biofilm static conditions were used and included a minimal medium (M9) [6 g of Na_2HPO_4 per litre, 3 g of KH_2PO_4 per litre, 1 g of NH_4CL per litre supplemented with 1 mmol l-1 of $MgSO_4$, 0.4% glucose and 0.4% casamino acids] (Poritsanos, 2005). To examin the impact of Epi, M9 was supplemented with a freshly prepared 50 and 100 µM/ml epinephrine (Epi) solution (Clarke et al., 2006).

PCR, cloning and sequencing experiments

The DNA of *E. coli* overnight cultures was obtained as described elsewhere (Sambrook and Russell, 2001). QS and biofilm-related genes were isolated by retrewing the sequences from GenBank at the NCBI web site, aligned using ClustalW 2 software (Larkin et al., 2007). Regions for PCR primer design were chosen based on previous studies as described in Table 7. Typically, the reactions were conducted in a thermocycler (BIORAD, CA, USA) with one step at 94°C for one minute; followed by 34 cycles at 94°C for one minute, 58°C for one minute, 72°C for one minute and a final extension step

at 72°C for two minutes. All PCR products were run in 1% agarose gels. PCR products were purified using Qiagen kits (Valencia, CA, USA), and cloning reactions were conducted using a TOPO kit according to the manufacturer's guidelines (Invitrogen). Cloned amplicons were purified and sent for sequencing at the University of Calgary, University Core DNA Services using an Applied Biosystems 3730 and 3730xl DNA sequencer.

In-silico analysis of UM146

Previous studies conducted by Sepehri et al., (2009) utilized a multi-locus sequences analysis (MLST) based on housekeeping genes and the fimbriae (*fimH*) gene in a set of human *E. coli* isolates. In this study, we focused on the UM146 strain which was compared with other *E. coli* strains whose genome had been completely sequenced and could be accessed from GeneBank. The UM146 *luxS, fimH, qseB,* and *qseC* genes were sequenced and aligned using BioEdit software (Hall, 1999). They were then compared with the completed genome of nine *E. coli* strains: DH10B, MG1655, W3110, HS, O157:H7_EDL933, O157:H7_Sakai, CFT073, UTI89 and APEC_01 (Accession numbers NC_010473, NC_000913, AC_000091, NC_009800, NC_002655, NC_002695, NC_004431, NC_007946, NC_008563) using BLAST (Tatusova and Madden, 1999). The *fimH* gene from AIEC LF82 was included for comparison purposes (Accession number AF288194.1). Aligned sequences were then used to construct Phylogenetic trees using MEGA version 4 (Kumar et al., 2001). The robustness of the trees was tested using the UPGMA method by bootstrapping each tree 1,000 times.

Isogenic mutations of UM146 *luxS*, *fimH*, *qseB* and *qseC* genes

Mutations were performed based on the lambda red technique (Datsenko and Wanner, 2000). Briefly, UM146 cells were electroporated with pKD46 plasmid. PCR products were obtained with primers homologous to sequences within the 5’ and 3’ ends of the target genes which were designed and used to replace the genes with a chloramphenicol-resistant gene derived from the template plasmid pKD3 (Table 7) (Datsenko and Wanner, 2000). Chloramphenicol (25 μg/ml) was used for the selection of mutant strains. Confirmation of the mutation was carried out by PCR and sequencing the mutated genes.

Table 7. Primers and plasmids used in manuscript II

Quorum sensing and biofilm related genes	Source
fimH-F 5'CTGGTCATTCGCCTGTAAAACCGCCA	Kotlowski *et al* 2007
fimH-R 5'GTCACGCCAATAATCGATTGCACATTCCCT	Kotlowski *et al* 2007
luxS-F 5' CTGACTAGATGTGCAGTTC	This study
luxS-R 5' ATGCCGTTGTTAGATAGCTTC	This study
qseB-F 5' GCGAATTTTACTGATAGAAGATGAC	This study
qseB-R 5' GCGTTCCTCAACCTGTGACAAACC	This study
qseC-F 5' CAACGTCTTAGTCTGCGAGTC	This study
qseC-R 5' GAAGTTACCAGCTTAC	This study
Lambda red mutation	
luxS:Cat-1	This study
5'CTGACTAGATGTGCAGTTCCTGCAACTT	
CTCTTTCGGCAGGTGTAGGCTGGAGCTGCTTC	
luxS:Cat-2	This study
5'ATGCCGTTGTTAGATAGCTTCACAGTCGAT	
CATACCCGGATGGAAGCGCCCATATGAATATCCTCCTTAGT	
fimH:Cat-1	This study
5'GTCACGCCAATAATCGATTGCACATCCCT	
GCAGTCACCTGTGTAGGCTGGAGCTGCTTC	
fimH:Cat-2	This study
5'CTGGTCATTCGCCTGTAAAACCGCCAATG	
GTACCGCAATCCCTATTGGCGCATATGAATATCCTCCTTAGT	
qseB:Cat-1	This study
5'TCATTTCTCACCTAATGTGTAGCCAATACCA	
TGCACGGTACGAATAAAATCGTGTAGGCTGGAGCTGCTTC	
qseB:Cat-2	
5'TGCGAATTTTACTGATAGAAGATGACATGCT	This study
GATTGGCGACGGCATCAAAAACATATGAATATCCTCCTTAGT	

qseC:Cat-1 5’CAACGTCTTAGTCTGCGAGTCAGGCTGACGCT AATCTTTTTAATTCTGGCCTCGGTGACCTGGCTGCT TTCCAGCTTTGTCGCCTGGAAACAAACAACGGA TAACGTCGAGTGTAGGCTGGAGCTGCTTC	This study
qseC:Cat-2 5’GAAGTTACCAGCTTACCTTCGCCTCAAATCCACCTT GTTCCGCATTCCCAAATTCAACATTCATGTCATGCAG CTTGGCGATTCGCTGGACAATCGATAGCCCAAGTC CCATATGAATATCCTCCTTAGT	This study
Plasmids	
PKD46	Datsenko and Wanner, 2000
PKD3	Datsenko and Wanner, 2000
pCR4-TOPO	Invitrogen

Static biofilm formation assay

E. coli strains were grown in LB medium at 37°C overnight in Falcon tubes until they reached an optical density (OD) of 600_{nm} between 0.8 and 1. The cultures were diluted 1:100 into the respective media: M9, M9 plus 50 µM Epi and M9 plus 100 µM Epi. A volume of 200 µl was dispensed in a 96-well microtiter plate of polyvinylchloride plastic (Becton Dickinson Labware). Negative wells contained media only, and each strain was tested six times in three different plates, obtaining 18 replicates per each strain. Plates were inoculated aerobically without shaking at 37°C for three different time periods: 12 hours, 24 hours and 48 hours. The cells were stained with the addition of 25 µl of 1% Crystal Violet (CV) solution to each well for 15 minutes. Plates were rinsed several times with H_2O to remove any unbound cell. Biofilm formation was quantified with the addition of 200 µl of 95 % ethanol. The volume was transferred to a new microtiter plate, and the OD was measured at 595_{nm} (A595) using a MicroPlate Reader (BIORAD, CA, USA). The readings were then corrected using the negative control (modified from Poritsanos, 2005).

Motility assay

The motility experiments were conducted based on a method described by Lane et al. (2005). Motility was measured using soft agar plates (1% tryptone, 0.5% NaCl, 0.25% agar) prepared a day prior to performing the assay. Overnight strain samples (400 µl) were used to inoculate 20 µl of LB broth and incubated at 37°C under shaking

conditions (200 rpm), until they reached an OD 600 nm of 1. Next the soft agar plates were stab-inoculated with the cultures and incubated at 30°C for 16 hours. The diameter of the bacterial swim zone was then measured. The experiments were conducted in triplicate during three separate days, obtaining nine measurements for each strain.

RNA extraction and real time RT-PCR experiment

Cultures of strains UM146, Δ*luxS* and Δ*fimH* were grown aerobically in LB medium at 37°C overnight. Cultures were diluted 1:100 in M9 medium or M9 treated with 50 and 100 μM epinephrine solution, and then placed into six-well plates to grow the biofilm for 12 hours at 37°C. Next, the liquid fraction was disposed of and the attached cells were re-suspended in a RNA preserving solution (Ambion). RNA was extracted from three replicate samples using an RNeasy Mini Kit as described in the manufacturer's protocol (Qiagen, Valencia, CA, USA). Conserved regions from the target genes were sequenced and sent to the TagMan Custom Centre for primer design (Assays-by-Design, Applied Biosystems) (Table 8). Real-time reverse transcription-PCR (RT-PCR) was performed using a high-capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Applied Biosystems).

The *qseC* and *flhD* mRNA levels were quantified using TagMan real-time PCR with a StepOne system (Applied Biosystems). The *rpoA* housekeeping gene product was used as an endogenous reference (Table 8). Standard curves were generated to determine the right amount of cDNA. The amplification conditions were two minutes at 50°C, ten minutes at 95°C, and a two-step cycle of 95°C for 15 seconds and 60°C for 60 seconds for a total of 45 cycles. Data were normalized to levels of *rpoA* and the expression level of

target genes was compared using the comparative Ct method (Anonymous, 1997). Real-time data are presented as a change in the expression levels compared to the wild type levels.

Table 8. Real –time RT-PCR probes and primers

RNA target	Function		Probe/primer sequence
rpoA	RNA polymerase subunit Alpha	Probe	5’-(FAM)-CTTTGCCCTGAACTCT-(NFQ)-3’
		F	5’-ACTGACTCTTCCGCAAATGGT-3’
		R	5-GCCAATGCCAGATTTATTCAAGGT-3’
flhD	Master flagella regulon	Probe	5’-(FAM)-AAGCTGGCAGAAACC-(NFQ)-3’
		F	5’-ACTGACTCTTCCGCAAATGGT-3’
		R	5’-ACGGAAGTGACAAACCAGTTGATT-3’
qseC	Adrenergic receptor for epinephrine	Probe	5’-(FAM)-CCTGCGGATCATCG-(NFQ)-3’
		F	5’-CCGAAGTTGCTCAGCTCTCT-3’
		R	5’-GAATGTAATTGGAGCAGCGCTTTT-3’

FAM = 5-Carboxyfluorescein
NFQ = non-fluorescent quencher

Statistical analysis.

Data obtained from static biofilm experiments, motility assays and RT-PCR were averaged and subjected to a paired student t-test using the Excel program, and considering the $P<0.05$ value as significant.

RESULTS

In silico analysis using quorum sensing genes in AIEC

By comparing *E. coli* UM146 phylogenetic trees, it can be observed that this bacterium is genetically related to the uropathogenic *E. coli* strains (CFT073, UTI89) and avian pathogenic *E. coli* (APEC-O1), and distantly related to the pathogenic O157:H7 or the K-12 strains (Figure 2). Bootstrapping supports the clustering in each tree branch with values greater than 60. The UM146 strain was originally isolated from a CD patient and is highly invasive in tissue culture cell lines. This strain does not have the toxins typical of enterotoxigenic or enterohaemorrhagic *E. coli,* and multilocus sequence typing indicated that AIEC represents a series of new sequence types (Kotlowski et al., 2007; Sepehri et al., 2009). Adherence to CEACAM6 is conducted possibly via *fimH,* and the sequencing of this gene places it in close proximity to *E. coli* LF82, a well-characterized AIEC strain (Figure 2A) (Sepehri et al., 2009).

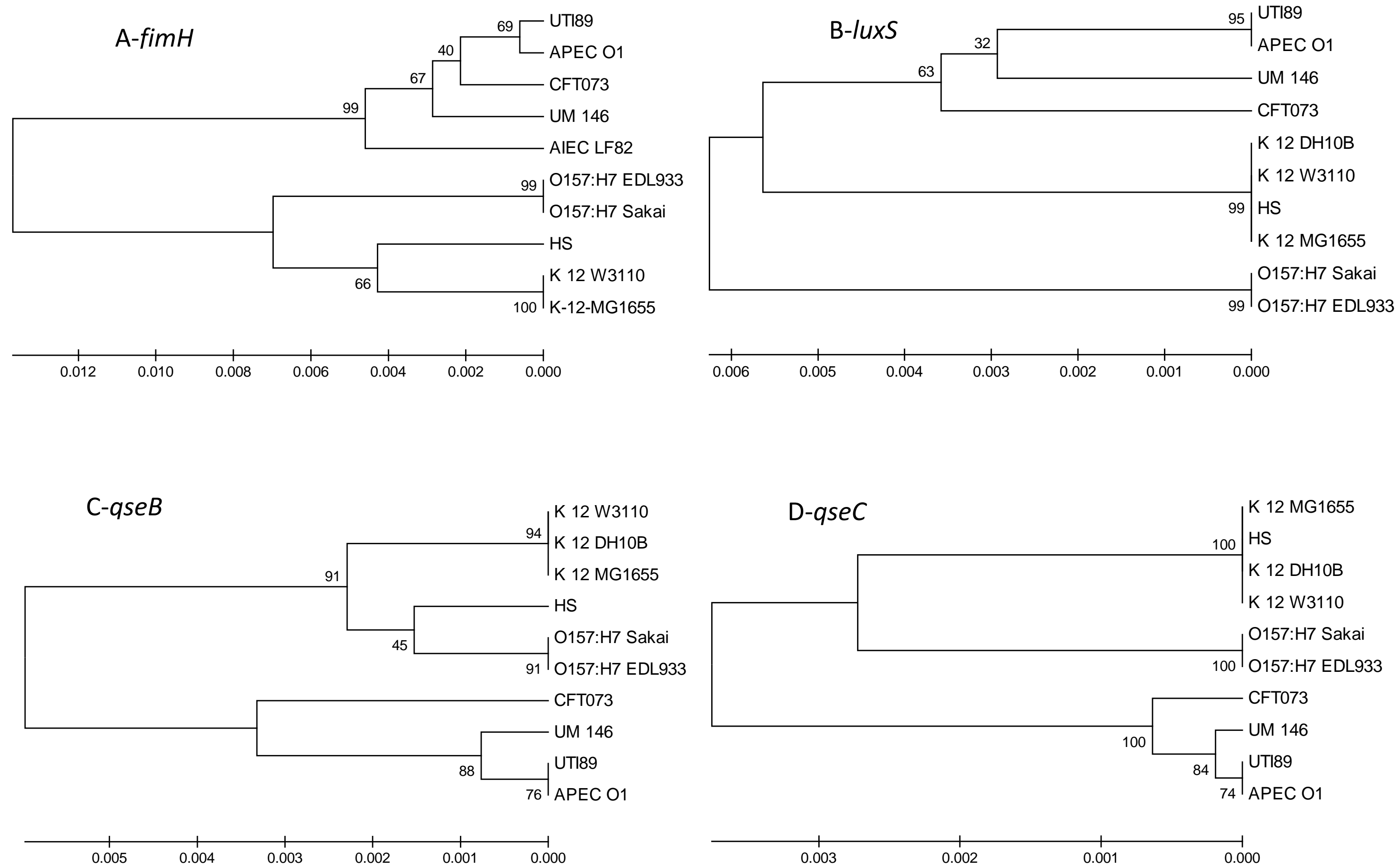


Figure 2. Phylogenetic trees of UM 146 and other *E. coli* strains based on sequences of *fimH* gene (A), *luxS* (B), *qseB* (C) and *qseC* (D). The Unweigthed Pair Group Method with Aritmetic Mean (UPGMA) method was used to create phylogenetic trees. The boostrap from 1000 replicates are taken to represent the phylogeny.

Mutations of *luxS*, and *fimH* genes did not affect biofilm formation by UM146

To determine the role of QS systems and type 1 pili on biofilm formation, mutations were developed affecting: 1) the production of autoinducers signals (*luxS*); 2) the two-component system operon genes *qseBC* that includes the adrenergic receptor (QseC) and its response regulator (QseB); and, 3) type 1 fimbriae adhesin synthesis in *E. coli* UM146 using the lambda red technique developed by Datsenko and Wanner (2000) (Table 7).

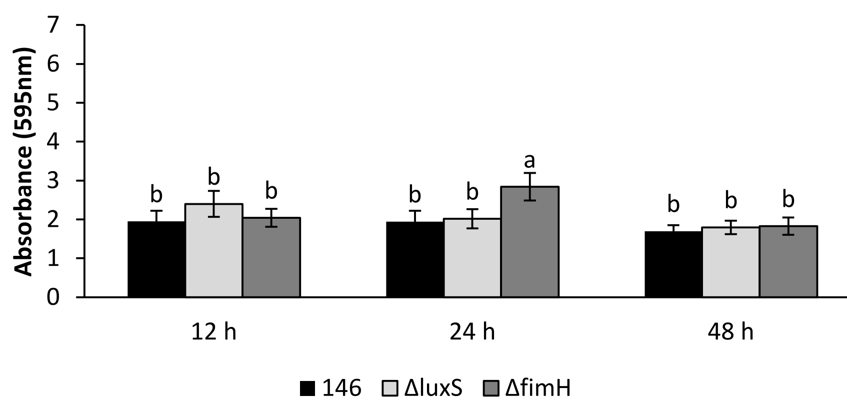
Biofilm formation experiments were conducted using a minimal medium (M9) supplemented with glucose (0.4%) and casamino acids (0.4%), which can yield the highest amount of biofilm in environmental *E. coli* strains (Reisner et al., 2006). Initially the biofilm analysis of UM146 wild type and mutants were incubated with M9 media for three different time spans: 12 hours, 24 hours and 48 hours (Figure 3A). The mutations of the *luxS* and *fimH* genes did not decrease biofilm formation of UM146 (Figure 3A). There was a significant reduction of biofilm in the Δ*luxS* mutant from 12 to 48 hours ($P< 0.05$), while Δ*fimH* significantly increased biofilm production for the first 24 hours ($P< 0.01$), after which biofilm formation was decreased from 24 to 48 hours ($P< 0.01$). The UM146 parental strain biofilm was not significantly affected the time allowed for biofilm development (Figure 3A).

In contrast, when the strains were supplemented with two different concentrations of the hormone epinephrine (Epi), 50 and 100 μM/ml, there was a substantial increase in the biofilm of the wild type and mutants (Figures 3B, 3C). The three strains produced

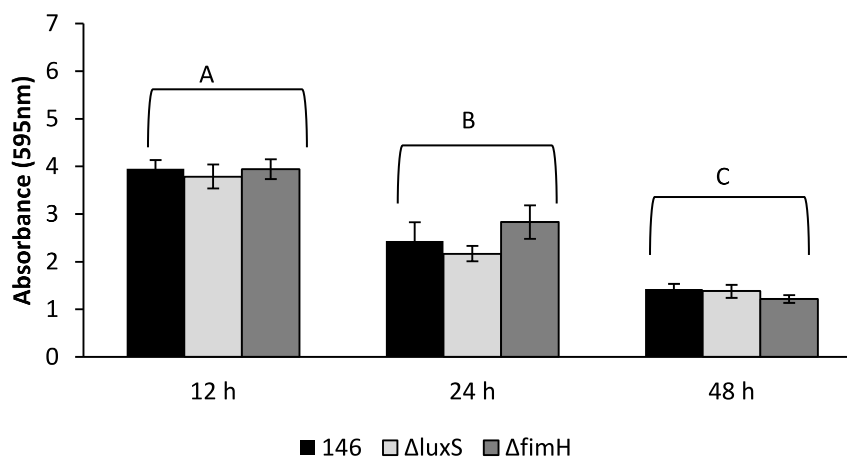
higher biofilm at the earlier stages (12 hours) than the later hours of 24 and 48 when 50 μM/ml-Epi was added. The 12-hour period yielded the highest absorbance values (3.95) for the strains, while at 48 hours the biofilm was reduced to approximately half (until it decreased to 1.4 absorbance). The t-test demonstrated a highly significant difference among the strains treated with the 50 μM/ml-Epi. All the strains differed in the time set with values $P < 0.01$ (Figure 3A).

The addition of 100 μM/ml Epi to M9 had the biggest impact in biofilm formation of the UM146 strains (Figure 3C). The time set showed an increase of biofilm at 12 hours for all the strains, followed by a sharp decrease at 24 hours and a slight increase at 48 hours (Figure 3C). The majority of the time comparison revealed a high statistical significance ($P < 0.01$), with one exception in the wild type group from 12 to 48 hours, and also one exception in the Δ*fimH* group from 24 to 48 hours (Figure 3A). The addition of Epi seems to have a dosage-dependent effect on the biofilm of the UM146 wild-type and mutants strains, especially at 12 hours (Figures 3B, 3C). It is noted that the biofilm formation ranged between 2, 4 and 5 absorbance values for the non-treated, 50 and 100 μM/ml-Epi groups respectively.

A- not treatment



B-plus 50µM Epi



C-plus 100µM Epi

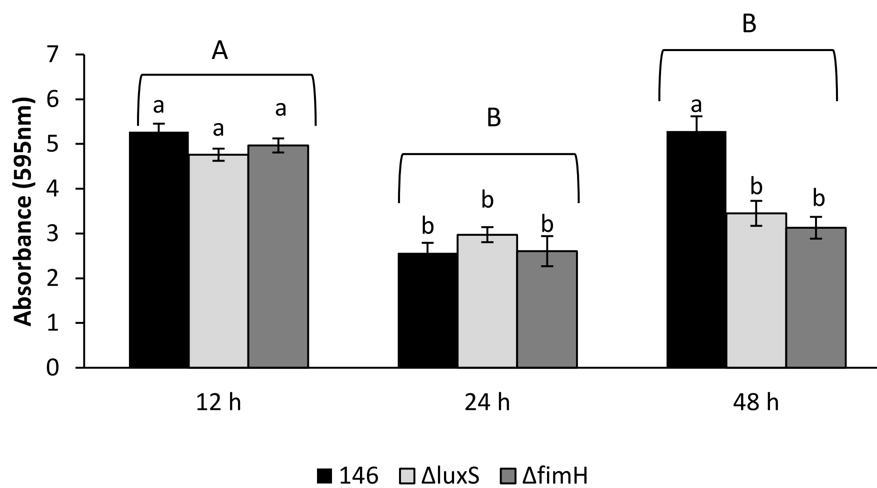


Figure 3. Time set course biofilm formation of *E. coli* UM 146 and mutants. (A) Biofilm formation in M9 medium supplemented with glucose (0.4%) and casaminoacids (0.4%). (B) Biofilm formation in M9 supplemented with 50µM epinephrine. (C) Biofilm formation in M9 supplemented with 100µM epinephrine. Subscripts with different letters indicate significant difference at $P < 0.05$. Capital letters indicate differences between time course, small letters indicate differences between strains.

***E. coli* gene expression of the adrenergic receptor (*qseC*) and the flagella master regulon (*flhD*) in the presence of Epi in a static biofilm.**

Using the Tagman chemistry we were capable of confidently quantifying the expression levels of two crucial genes (*qseC, flhD*) involved in the cascade regulation of the QS system. All data is reported as relative quantification values based on the expression level of the UM146 parental strain (Figure 4). It is observed that the mutations in the *luxS* and *fimH* genes did not down-regulate the expression levels of either the *qseC* or *flhD* genes. An additional note is the positive effect of epinephrine (Epi) at both levels (50 and 100 µm/ml) over the gene expression of *qseC* and *flhD* in 12 hours of biofilm formation in all the strains. In particular, the most significant effect was the use of the highest Epi concentration (100µm/ml) whereby the gene expression was increased in comparison to non-treated group in the *luxS* and *fimH* mutations (Figure 4). Thus this is clear evidence that the AI-2 QS system, where the molecule is synthesized by *luxS*, does not control the expression of *qseC* and *flhD* and both genes were positively stimulated by the hormone Epi.

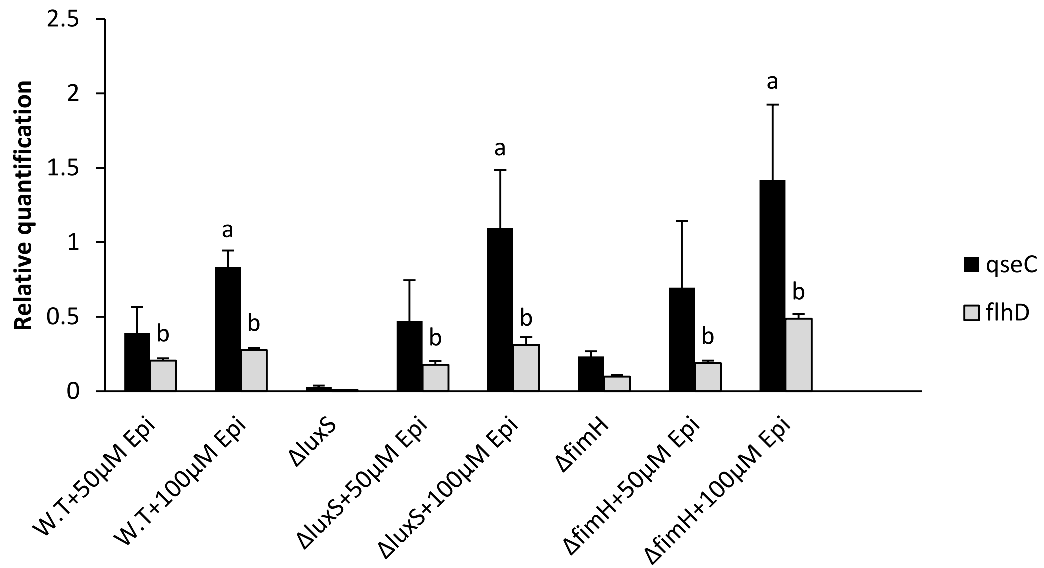


Figure 4. *E. coli* UM 146 and derivate strains expression of quorum sensing and flagella related genes at 12 hours biofilm development. All the data is represented as relative quantification relative to the wild-type (WT) parental strain UM146 with a relative quantification value of (1). Student t-test was conducted comparing the treated group against the untreated. Subscripts indicate significance difference at $P < 0.05$.

Motility in AIEC 146 was affected by mutating the *qseBC* operon.

In order to determine the bacterial ability to swim, the wild type UM146 and its isogenic mutants' motility were assessed for their motility in the presence or absence of epinephrine (50 μM/ml) (Figure 5). Initialy, motility was not significantly affected between parental UM146 and the mutated strains (Δ*luxS*, Δ*fimH* and Δ*qseB*) at 16 hours of growth (Figure 5). In contrast, the motility decreased substantially upon mutation of the adrenergic receptor gene *qseC*. Also, it is worth noting that motility significantly increased within strains by adding Epi, especially in the Δ*luxS*, and Δ*fimH* mutants, but adding Epi did not significantly increase the motility in the Δ*qseB* and Δ*qseC* mutants. This research provides further evidence of the positive effect of Epi over the AIEC motility which is controlled by the *qseBC* operon.

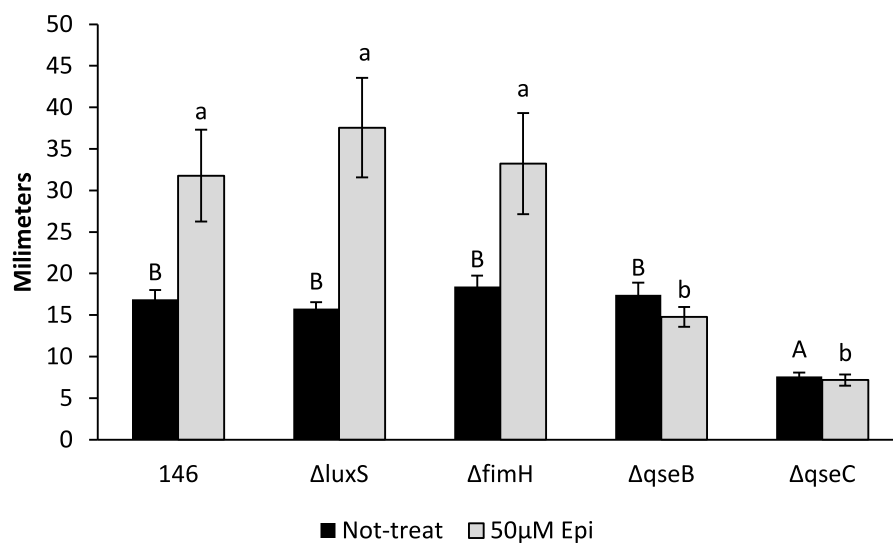


Figure 5. Motility assay of UM 146 at 16 hours. Subscripts with different letters indicate significance difference at P< 0.01. Capital letters indicate differnces between not-treatment strains; small letters indicate differences between treatment with Epi.

AIEC UM146 biofilm formation is affected by mutating the *qseBC* operon

Due to the significant impact of the *qseBC* operon on the AIEC motility, we were interested to know if similar results could be obtained in a biofilm assay. As observed in Figure 6, we found a trend for biofilm formation in the wild type (146) and two isogenic mutants (*luxS* and *fimH)* where there was an enhancement of biofilm by adding Epi in a dosage dependat manner. However, the same situation was not found in the *qseB* and *qseC* mutants. In fact, it was initially observed that mutations carried over the operon made the bacterium form twice as much biofilm (with an absorbance value of 6) compared with the parental strain (an absorbance value 1.98). Interestingly the addition of Epi did not seem to have an impact over the biofilm of the *qseC* and *qseB* mutants. Indeed, the addition of either 50 or 100 μM Epi did not significantly enhance the formation of biofilm in both mutant strains.

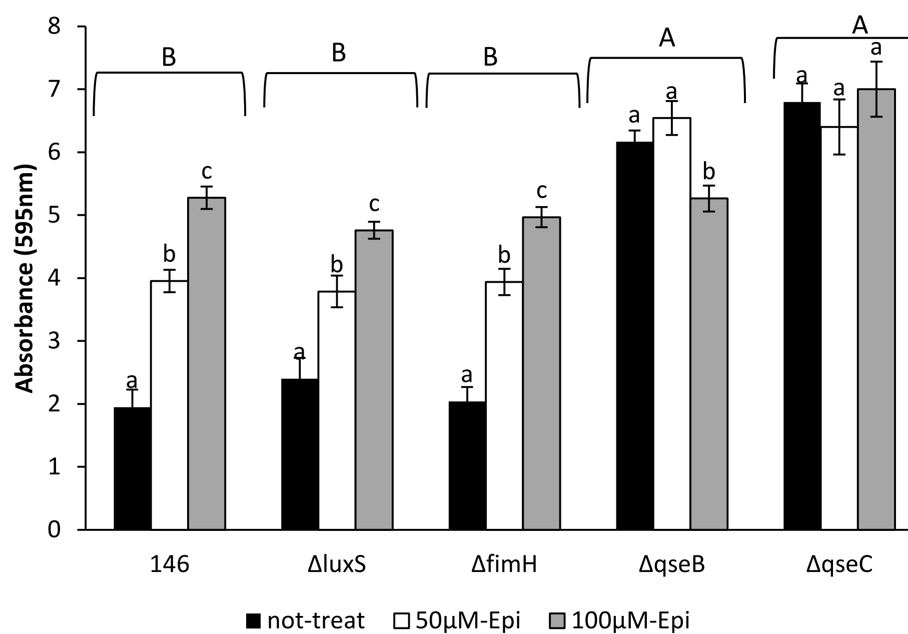


Figure 6. Biofilm formation of UM 146 in M9 plus epinephrine at 12 hours. A Students T-test was conducted to compare the untreated group against the treatment groups. Capital letters are used to indicate differences between strains; small letters are used to denote differences between treatments within the same strain ($P<0.05$).

DISCUSSION

The Epi and Ne system of QS was first described by Sperandio et al. (2003) in *E. coli* O157:H7 when investigating the expression of genes in the locus of attachment and effacement (LEE). This new autoinducer system was called AI-3/Epi/Ne (Sperandio et al., 2003). But in *E. coli* the AI-3 system is not the only mechanism of QS, and the AI-2 system has been extensively studied in the non-pathogenic *E. coli* K-12 (Wood, 2009). The autoinducer in AI-2 is a furanosyl borate diester (Chen et al., 2002) and is synthesized by the product of the *luxS* gene (Surette et al., 1999). *LuxS* is involved in the detoxification of S-adenosylmethionine (SAM), and converts ribosehomocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione, a precursor of AI-2 (Schauder et al., 2001).

To further evaluate the potential role of *luxS*, which is present in AIEC, we created a *luxS* mutant. Absence of *luxS* did not effect biofilm formation (Figure 3), but when Epi was added to the medium at either 50 μM (Figure 3B) or 100 μM (Figure 3C), there was an increase in biofilm formation. Sperandio et al. (2003) initially suggested that *luxS* was involved with AI-3 autoinducer production because *luxS* mutants could not produce AI-3. Walters et al. (2006) subsequently demonstrated that a *luxS* mutation decreased the AI-3 production in O157:H7, and it was proposed that *luxS* is associated with the sulphur amino acid metabolism in the bacterium. In addition, motility was unaffected by mutating *luxS* but was increased when adding Epi. We thus suspected that AIEC does not regulate biofilm formation and motility via the genes associated with the AI-2 system including *luxS*.

In *E. coli* O157:H7 the AI-3 molecule is involved in the activation of the locus of the enterocyte and effacement (LEE) and flagellar genes (Clarke and Sperandio, 2005b; Pacheco and Sperandio, 2009). Although the structure of the AI-3 molecule is not known, it is probably an aromatic compound with structural similarities to Epi and Ne as these hormones appear to bind the same bacterial receptor. The gene products of *qseBC* form a two-component signal transduction system, which after sensing AI-3/EPI/NE the sensor kinase QseC is autophosphorylated, leading to the transduction of the signal to the regulator QseB, which then results in the transcription of the master flagella regulon *flhDC* (Clarke et al., 2006; Hughes et al., 2009).

In *E. coli* O157:H7, the LEE is regulated by AI-3 with Epi/Ne inducing the transcription of the LEE locus. If *qseC* is mutated, the LEE may not be transcribed in the presence of Epi/Ne (Hughes et al., 2009). However, AIEC UM 146 does not have a LEE and the best described adherence factor is *fimH*, which is the terminal subunit adhesin of the type I pilus structure (Sepehri et al., 2009). It is known that *fimH* has a structure which is conserved among AIEC and some urinary tract isolates (CFT073) and some avian pathogenic *E. coli* (APEC O1) (Figure 2A) (Sepehri et al., 2009). There is a host-specific attachment by *fimH* to CEACAM6 in humans, and if *fimH* is mutated AIEC will reduce adherence and invasion to epithelial cells (Glasser and Darfeuille-Michaud, 2008). We demonstrated that *fimH* mutations do not result in a reduction in biofilm formation even in the presence of Epi (Figure 3).

To evaluate the potential relationship between Epi in wild type and *luxS* and the *fimH* mutation, we investigated the effects on the flagella regulon. In *E. coli* O157:H7

Epi/Ne/AI-3 is recognized by QseC which leads to a series of events that up-regulated the *flhDC*, which are members of the flagellar regulon (Hughes et al., 2009; Sperandio et al., 2002). We demonstrated that *qseC* and *flhD* genes are up-regulated in the presence of Epi and their expression worked in a dose-dependent manner (Figure 4). This response was independent of *luxS* or *fimH* mutations. These observations are in agreement with Clarke et al. (2006) and Pacheco and Sperandio (2009).

The flagella regulon influences motility, and the *luxS* and *fimH* mutation did not affect motility in the presence of Epi compared to the wild type controls (Figure 5). However, when we mutated *qseB* or *qseC* there was no increase in the bacterial motility in the presence of Epi (Figure 5). Hughes et al. (2009) mutated *qseB* and *qseC* in *E. coli* O157:H7 and measured motility in the presence of AI-3. They found no effect of AI-3 in the *qseB* mutant but a reduction of motility in the *qseC* mutant. Clarke et al. (2006) found no effect of Epi on the motility in *qseC* mutants of *E. coli* O157:H7. Thus it is clear from our results the AIEC strain UM146 responds to Epi which affects motility.

The Epi/Ne/AI-3 system of autoinducer signalling is likely to be fairly widespread in enteric bacteria (Pacheco and Sperandio, 2009). In *E. coli,* the greatest genetic similarity probably lies between AIEC, avian pathogenic *E. coli* and urinary tract *E. coli* (Figure 2). The phylogenetic relationships between *fimH*, *luxS, qseB* and *qseC* indicate a high level of similarity, suggesting a common evolutionary origin. The urinary tract pathogen *E. coli* CFT073 is known to form a biofilm in the urinary tract (Allsopp et al., 2010) and avian pathogenic *E. coli* appears to be highly adapted to biofilm formation (Skyberg et al., 2007). In this report, we found that mutating *qseC* and *qseB* did not

decrease the biofilm formation ability of our AIEC strain. In fact, the opposite situation happened, showing that the mutants were capable of producing a greater amount of biofilm than the parental strain. Further, addition of Epi did not enhance AIEC biofilm formation. In this situation, we suspected that perhaps more than one biological pathway can be involved in the AIEC biofilm phenomenon (Pruss et al., 2006), which could predominate in the absence of the *qseBC* operon.

CONCLUSION

Unlike previous studies examining the role of AI-2 QS on *E. coli* K-12 biofilms, this study is unique in that it examined an *E. coli* strain whose niche might be specific for immune deficient CD patients. We reported that AIEC contains the gene responsible for producing the AI-2 molecule (*luxS*) but its effect did not appeared to be involved in bacterium motility and biofilm formation. In addition, we found that motility and biofilm were positively affected by the gut hormone epinephrine, meaning the novel QS system (AI-3/Epi/Ne) seems to have a role in controlling both phenomena. Nonetheless, it is suspected that other pathways may have a regulatory role in the AIEC biofilm; a hypothesis worth of further exploration.

Manuscript III

Impact of the autoinducer 3-epinephrine-norephineprine quorum sensing system over gene expression and cell invasion in a biofilm model of adherent and invasive *E. coli* (AIEC).

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ABSTRACT

E. coli utilize biofilm formation to adhere to inert and live surfaces and thus it expresses several pathogenic mechanisms in clinical strains. Adherent and invasive *E. coli* (AIEC) are novel pathogenic classes implicated with inflammatory bowel diseases, ulcerative colitis (UC) and Crohn's Disease (CD). The clinical strain UM146 was obtained from a case of CD. The gene expression in a static biofilm in a minimal medium supplemented with the hormone epinephrine (Epi) using microarrays, plus the ability to invade epithelial HT-29 cells, was evaluated. Also, isogenic mutants were constructed in the *qseBC* operon involved in the AI-3/Epi/Ne QS system to determine its degree of significance in the AIEC biofilm and invasion. We report that this QS system exerts divergent effects on AIEC biofilm formation and invasion of HT-29 cells. Deleting the adrenergic receptor *qseC* did not decrease the UM146 biofilm but significantly reduced invasion of HT-29 cells. Using microarray analysis, it was observed that significant levels of expression occurred between the parental strains and the isogenic mutations in *qseB* or *qseC* genes using minimal medium conditions supplemented with Epi. We report that several gene functions were affected by both mutations, including the flagella and fimbria network, and the iron acquisition systems which were dependent on the presence of Epi and needed for biofilm formation. However, other gene functions such as the osmolarity system and cellulose synthesis were not dependent on the hormone presence since they continued to be active in both mutations. In-vitro invasion experiments concur that adding Epi increased intracellular bacterial numbers in the

parental strain, but mutation in its adrenergic receptor *qseC*, significantly reduces the numbers. However, the numbers were not reduced entirely. Taken together these results suggest the AI-3/Epi/Ne system can regulate a large proportion of genes dictating AIEC ability to swim and attach to a surface. Nevertheless, in the absence of a sensor molecule receptor like *qseC*, AIEC may use alternative pathways to counter this absence and continue forming biofilms.

Key words: AIEC *E. coli*, biofilm formation, epinephrine, microarrays

INTRODUCTION

Biofilms allow bacteria to adhere to biotic and abiotic surfaces where bacteria use this mechanism to prevail against antimicrobial treatments and the host immune system (Costerton et al., 1999; 1987). In *Escherichia coli* biofilm formation, there are several developmental stages, including: (i) reversible attachment of planktonic cells to a surface, (ii) irreversible attachment, (iii) architecture development of microcolonies, (iv) maturation of biofilm, and (v) dispersion of planktonic cells from the biofilm (Van Houdt and Michiels, 2005). In *E. coli,* it has been demonstrated that different biofilm stages involve an ancillary of diverse genes which can be regulated up or down depending on the biofilm growth stage, the culture conditions, and the strain involved (Hancock and Klemm, 2007; Wood, 2009).

Microarray technology has been used in several studies, providing insight into global gene regulation including the flagella-motility network, fimbria synthesis, polysaccharide-matrix, two-component phosphotransfer system among others, both at planktonic and biofilm stages in *E. coli* (Kendall et al., 2007; Hancock and Klemm, 2007; Pruss et al., 2006). Although it is difficult to compare biofilm versus planktonic studies even within biofilm experiments, a large number of genes described as useful in planktonic situations are involved in earlier stages of biofilm formation (Beloin et al., 2008).

The flagella-motility system is required for *E. coli* to swim in the growth medium (Beloin et al., 2008). In K-12 strains it has been demonstrated that flagella are required for biofilm formation although not completely essential, since mutants deficient in

flagella synthesis are still capable of forming a biofilm (Pratt and Kolter, 1998; Wood, 2009). In pathogenic *E. coli* strains, biofilm studies have shown variable results in the flagella-motility gene expression profile showing up and down regulation of those genes in uropathogenic and O157:H7 strains (Bansal et al., 2007; Hancock and Klemm, 2007). However, flagella-motility is only one step in the process of biofilm formation as attachment to any kind of surface is realized via the fimbriae, and activation of such organelles is directly proportional to a surface to which the bacterium will be anchored (Knight and Bouckaert, 2009). Once the bacteria is attached, another set of genes are activated, building up the matrix which can be composed of several polysaccharides including cellulose. Even though the role of cellulose is not well understood, it may confer a barrier of protection against hostile conditions (Beloin et al., 2008). Importantly, the majority of these biological aspects in the planktonic and biofilm growth of *E. coli* can be regulated by signals present in the environment involving sophisticated transmission systems.

Quorum sensing (QS) systems are used by bacteria to control biological processes including biofilm formation (Beloin et al., 2008). In *E. coli,* two QS systems have been described in detail. Autoinducer 2 (AI-2) needs the presence of the *luxS* gene to be synthetized (Xavier and Bassler, 2005), and the impact of AI-2 on the biofilm formation in K-12 strains has been extensively studied (Wood, 2009). The AI-3/Epi/Ne system, studied primarily in O157:H7 involves an unknown molecule named autoinducer 3 (AI-3) as well as the cathecholamine hormones epinephrine (Epi) and norepinephrine (Ne). This system serves as a cross-talk mechanism between the bacterium and its host (Parker

and Sperandio, 2009; Hughe and Sperandio, 2008). As described in manuscript II, the AI-3/Epi/Ne system works over the operon *qseBC,* regulating flagella synthesis in O157:H7 and K-12 (Sperandio et al., 2002; Kendall et al., 2007). In addition, the two hormones, Epi and Ne, have a regulatory role over biofilm formation and attachment to cell lines in O157:H7 under in-vitro conditions (Bansal et al., 2007). Moreover, the AI-3/Epi/Ne system is capable of controlling several other genes involved in virulence and iron uptake via this two-component system (Hughes and Sperandio, 2008).

Adherent and invasive *E. coli* (AIEC) are pathogenic strains associated with gastrointestinal affections, ulcerative colitis and Crohn's Disease (Sartor, 2008). Several studies demonstrate that flagella and fimbriae are crucial factors for the AIEC invasion of epithelial cells (Barnich et al., 2003; Claret et al., 2007). Our previous study (manuscript II) demonstrated that a clinical isolate of AIEC regulates the flagella-motility network via the AI-3/Epi/Ne system. Adding Epi to the culture media increased the static biofilm in a dosage-dependent manner. Nonetheless it was observed that the formation of biofilm was not decreased once mutations of the *qseB* and *qseC* genes were produced. Additionally, supplementing Epi did not significantly increase the biofilm in these mutants. Therefore the focus of this investigation relied upon determining changes in gene expression in the *qseB,* and *qseC* AIEC mutants supplemented with Epi in a static biofilm situation. Furthermore, the effect of several AIEC mutants over the invasion process in cell lines was evaluated. The two-component system operon *qseBC* can control many genes involved in biofilm formation like flagella and the fimbriae network, and can also partially control the AIEC invasion of cell lines.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Adherent and invasive *E. coli* UM146 was obtained from our lab collection (Kotlowsky et al., 2007). The isogenic mutants Δ*fimH*, Δ*luxS*, Δ*qseB* and Δ*qseC* were generated previously using the lambda red technique described by Datsenko and Warner (2000). Luria-Bertani medium (LB) supplemented with 0.4% (wt/vol) glucose was used for overnight cultures. Minimal medium (M9) [6 g of Na_2HPO_4 per litre, 3 g of KH_2PO_4 per litre, 1 g of NH_4CL per litre supplemented with 1 mmol l^{-1} of $MgSO_4$, 0.4% glucose and 0.4% casamino acids] was used for the biofilm experiments, and epinephrine was purchased from the Sigma Chemical Co. (St. Louis, MO).

Static biofilm formation assay

Cultures of strains UM146, Δ*qseB* and Δ*qseC* were grown aerobically in LB medium at 37°C until an OD_{600} ~1 was reached. Cultures were diluted 1:100 in M9 medium and M9 was treated with a freshly prepared 100 µM Epi solution. Both were placed in six-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) where the biofilm grew for 12 hours at 37°C statically. Next the liquid fraction was disposed of and the attached cells were resuspended in an RNA preserving solution (Ambion), and the suspended solution was kept at -80°C until further use.

RNA isolation and DNA microarrays

Biofilm cultures were harvested from the six-well plates obtained previously. Total RNA was isolated using TRIZOL reagent (Invitrogen). Briefly, biofilm cultures were centrifuged and the supernatant removed to add TRIZOL, and the mixture was incubated at 65°C for 10 minutes. Chloroform was added, and the mixture was centrifuged to separate the upper phase and mixed with isopropanol to precipitate the RNA. The RNA was washed with 70% ethanol twice and dried at room temperature to be dissolved in Rnase free water. The RNA obtained was digested with Dnase I according to the manufacturer's protocol, and further purified using kit columns (Qiagen). The RNA was re-suspended in Rnase free water and the RNA quantity and quality was measured by spectrophotometry.

cDNA synthesis and labelling were performed according to the manufacturer's recommendation (Affymetrix, Santa Clara, CA). Ten µg of RNA was dissolved in 20 µl of RNase, DNase and pyrogen-free water (Gibco) and supplemented with 2 µl of the GeneChip Eukaryotic poly-A RNA control kit (Affymetrix, Santa Clara). Conversion to cDNA was done using SuperScript II and random hexamer primers (Invitrogen, CA). RNA was removed using 20 µl of 1N NaOH and incubated at 65 ^{0}C for 30 minutes. cDNAs were purified using MinElute PCR purification columns and the product was eluted in EB buffer (Qiagen). Fragmentation was carried out using DNAse I at 0.6U/ µg (Invitrogen, CA) using a cDNA quantity between 3 and 7 µg, and the mixture was incubated at 37 ^{o}C for 10 minutes. The DNAse was inactivated by heating the reaction at 98 ^{o}C for 10 minutes. Terminal labelling was performed using GeneChip DNA labelling

reagent (Affymetrix, Santa Clara) at 7.5 mM and 60U of terminal deoxynucleotidyl transferase (Promega, Madison, WI) at 37 ^{o}C for 60 minutes, and 2 µl of 0.5M EDTA (Sigma) was added to terminate the reaction. Hybridization was conducted using the Affymetrix GeneChip *E. coli* Genome 2.0 array, and performed at the Génome Québec Innovation Centre (McGill University, Montréal, Canada).

Invasion assays with HT29 intestinal cells

E. coli UM146 and its isogenic mutants Δ*fimH*, Δ*luxS*, Δ*qseB* and Δ*qseC* were tested for their ability to invade the intestinal cell line HT29. Monolayers of HT-29 cells were maintained in RPMI 1640 medium (Gibco) at 37 ^{o}C, 5% CO_2 supplemented with 10% (vol/vol) fetal calf serum (Invitrogen). Cells were seeded onto a 12 well tissue culture plate (Corning/Costar, USA) at approximately 4 x 10^5 cells per well, and incubated at 37^{0}C in RPMI till forming a monolayer. Monolayers were washed thrice with a 1X PBS solution. For experiments involving exposure of cells to live bacteria, the strains were grown in LB plus 0.4% glucose medium to obtain a concentration of approximately 1 x 10^7 CFU/ml in RPMI medium, and each suspension was applied to the 12 wells plates. Additionally, epinephrine (Sigma) was added at a concentration of 50 µM. After three hours, the medium was removed and the cells were washed five times with PBS. Invasion test cells were incubated with RPMI containing gentamycin (100 µg/ml) for one hour, then monolayers were washed in PBS five times and cells were lysed by vigorous pipetting 200 µl of 1% Triton X-100 in sterile water. Bacteria released from cells were serially diluted in peptone water solution (Gibco); aliquots were plated on LB-agar in triplicates incubated at 37°C and counted after 24 hours. All of the

invasion experiments were conducted twice independently obtaining a total data set reading of six values per strain.

Analysis of microarray data

Raw array data was processed using the FlexArray 1.5 software (Blazejczyk et al., 2007). Data normalization and background–adjustment was conducted using the robust multiarray average algorithm (RMA) (Irizarry *et al.,* 2003). The robustness of the data was enhanced applying the EB (Wright and Simon) algorithm and p-value ($P<0.05$) calculation (Blazejczyk et al., 2007). Due to the fact that RMA possesses higher sensitivity and specificity, it compresses the fold change (FC) value, and we reported this FC in log (base 2) considering a range between 0.5 and 2 as significant (Irizarry et al., 2003_b). A functional classification of gene groups was created based on the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (Dennis et al., 2003; Huang et al., 2009).

Student t-test

The colony forming unit (CFU) data obtained from the invasion assay was log (base 10) transformed, and the comparisons between groups and within treatments was conducted using a paired student *t* test where appropriate.

RESULTS

Effect of mutations Δ*qseB*, Δ*qseC* and addition of epinephrine on the AIEC biofilm formation gene expression profile

Our previous study showed that mutations of the *qseBC* operon did not decrease biofilm formation by UM146, but motility was reduced particularly in the Δ*qseC* mutant. In addition, it was observed that Epi did not significantly enhance the biofilm produced by both mutants (Figure 7). Because of these significant changes it was decided to conduct a static biofilm experiment on *E. coli* UM146 and the isogenic mutants Δ*qseB* and Δ*qseC* to evaluate the addition of Epi at 100 μM and determine their gene expression profile. Using the GeneChip *E. coli* 2.0 array from Affymetrix, the AIEC parental strain and mutants can be compared against four completed *E. coli* genomes including K-12, CFT073, O157:H7 St. EDL933 and O157:H7 St. Sakai. Since UM146 belongs to a novel phylogenetic group and its closest genetically-related strain is the uropathogenic CFT073 (Sepheri et al., 2009), the focus was on the microarray analysis based on the CFT073 genome. Initially our results yielded almost 3,000 genes and up to 1,000 were annotated as hypothetical proteins with unknown functions (data not shown). The DAVID database was used to classify the functional genes into groups and many of these included anion uptake, catabolism, and citric acid cycle to name a few (see appendix).

The biofilm-related genes were clustered into five groups including the two-component system, iron transport, cellulose synthesis, fimbrial synthesis and flagella genesis. The majority of the genes in those groups were regulated by the *qseBC* operon, indicating its importance over crucial biological functions in AIEC. The exception were the genes involved in cellulose synthesis where they remained up-regulated despite the presence of mutations in the *qseB* and *qseC* genes (Table 9-12a).

Genes involved in two component systems

Bacteria contain signalling systems that allow them to elicit adaptive responses to their environment. Adding Epi to the UM146 biofilm maintains an active majority of genes involved in the two-component system with fold changes (FC) ranging from 0.5 to 1.5 in the microarray comparisons (Table 9). The genes that have a fold change higher than 1 include those encoding outer membrane proteins (*ompC, ompG*), a transport activator (*pgtA*), multidrug efflux system (*yegN*), potassium-transporting enzymes (*kdpA, kdpB*) and sensor proteins (*kdpD*). The remnant genes with a fold change lower than 1 included some transcriptional regulator proteins (*basR, hydG*), histidine kinase (*citA, torS*) and the multidrug efflux system (*yegM, yegO*). The *qseC* and *qseB* mutations markedly decreased the gene expression profile, independent of the Epi addition. The Δ*qseC* mutation down-regulated more than half of the genes involved in the two-component system, including many sensor proteins and the multidrug efflux system. The exceptions were a few genes like *ompC, ompG*, *kdpB, htrA, evgS, baeS* and *citC* that remained up-regulated with fold change values ranging between 1.3 and 0.2. The Δ*qseB*

mutation resulted in larger numbers of genes which were up-regulated with respect to Δ*qseC*. The list included outer membrane protein (*ompC*), multidrug efflux system (*yegM, yegN*), nitrogen regulator protein (*glnG*), and potassium-transporting genes (*kdpA, kdpB*), and the fold change values that were not greater than 1. Importantly, the outer membrane porin protein, *ompC*, remained up-regulated in all cases.

Table 9. Gene expression comprising the two component systems in adherent and invasive *E. coli* (AIEC).

Gene function	Gene title	Fold change log2		
		146 vs 146+Epi	Δ*qseC* vs. Δ*qseC*+Epi	Δ*qseB* vs. Δ*qseB*+Epi
Outer membrane porin protein C	ompC	1.36	1.38	0.43
Outer membrane protein G precursor	ompG	1.13	0.11	-0.14
Sensor kinase dpiB	citA	0.81	-0.55	-0.47
Transport activator	pgtA	1.23	-0.37	NS
Glutamine synthetase	glnA	0.86	-3.56	-1.06
Multidrug efflux system subunit MdtA	yegM	0.82	NS	0.41
Multidrug efflux system subunit MdtB	yegN	1.45	-0.40	0.64
Multidrug efflux system subunit MdtC	yegO	0.51	NS	-0.19
Nitrogen regulation protein NR(I)	glnG	0.78	-0.10	0.27
Potassium-transporting ATPase subunit A	kdpA	1.11	-0.29	0.28
Potassium-transporting ATPase subunit B	kdpB	1.39	0.23	0.65
Serine endoprotease	htrA	1.58	0.31	NS
Putative transport sensor protein	c5041	1.54	-0.34	-0.23
Regulatory protein	pgtB	1.31	-0.22	NS
Sensor protein evgS; Sensor protein	evgS	NS	0.77	NS
Hybrid sensory histidine kinase TorS	torS	0.84	-0.18	-0.59
Sensor protein KdpD	kdpD	1.56	-0.20	0.14
DNA-binding transcriptional regulator BasR	basR	0.50	-0.15	-0.60
Sensor protein; Signal transduction Histidine-protein kinase baeS	baeS	NS	0.77	-0.32
Transcriptional regulatory protein ZraR	hydG	0.74	-0.56	0.13
Citrate lyase synthetase (citrate (pro-3S)-lyase ligase	citC	0.85	0.34	0.32
Hypothetical protein	c0784	1.31	NS	0.20
Putative Transcriptional regulatory protein	c5040	0.67	0.57	-0.40

NS (not significant)

Effects on expression of fimbrial and flagella genes

Swimming and attachment are crucial steps in biofilm, thus it was desirable to explore the effect of Epi and *qseBC* operon mutations on fimbriae and flagella-related gene expression. In general, Epi seemed to have an impact over expression of many flagella-fimbriae-related genes in AIEC (Table 10). Notably, adding Epi to the UM146 biofilm up-regulated fimbrial genes such as type 1 fimbria (*fimA, fimI*), F1C fimbrial subunit (*focA, focC,focD, focF*), S fimbrial subunit (*sfaD, sfaB, sfaC*) and Pap proteins (*papA,C,E,G,H,I,JK*) resulted in FC values ranging between 0.5 and 1.1. Other fimbrial genes which were annotated as putative or hypothetical proteins appeared up-regulated (Table 11). The Δ*qseC* and Δ*qseB* mutations increased the number of down-regulated fimbrial genes. The Δ*qseC* mutation-affected genes included type I fimbrial protein (*fimA*), F1C fimbrial subunit (*focA, focD*), S fimbrial regulatory protein (*sfaC*) and Pap proteins (*papA, papH, papG* and *papK*). Other putative fimbrial proteins (*yadC, yfcV*) and hypothetical proteins (*yadK, yadL*) were down-regulated as well (Tables 10, 11). Nonetheless there were some fimbrial genes in Δ*qseC* that remained significantly up-regulated, including *fimI*, *focF*, *papE* and *papI* with FC values of 0.55, 0.77, 1.12 and 0.59 respectively (Table 10). Similarly, Δ*qseB* down-regulated many fimbrial genes in the biofilm. However, there is a controversy within results that some genes like *focC* which appeared down-regulated in the Δ*qseB* mutation (FC value -0.16) were up-regulated in the Δ*qseC* mutation. The opposite situation happened with *focD, papA, papG, yadC, yehB* and *yadK* genes in Δ*qseC* and Δ*qseB* respectively (Tables 10, 11). This discrepancy in the expression of fimbrial genes during biofilms has been noticed in other

studies, even though the strains and growth conditions used in our experiments were different.

It has been long known that flagella synthesis is a key element in *E. coli* biofilm formation. Our results show that all of the genes involved in flagella synthesis were up-regulated by adding epinephrine in the UM146 array with FC values greater than 0.5 (Table 11). In contrast, both mutations in the *qseBC* operon negatively affected the expression of several genes grouped in the flagella synthesis cluster. In particular Δ*qseC* down-regulated many flagella-related genes with the exception of *fliR* that remained up-regulated (FC 0.4). Moreover, Δ*qseB* down-regulated a large number of flagella-related genes but there were a higher number that remained active compared to Δ*qseC*; such genes included *flhE, fliC* and *fliH* with FC values of 0.4, 0.6 and 0.28 respectively (Table 11). This down-regulation trend in flagellar genes has been studied in other investigations, revealing the role of the *qseBC* operon in expression of *E. coli* flagella synthesis.

Table 10. Fimbrial related gene expression in AIEC biofilms

Gene function	Gene title	Fold change log2		
		146 vs 146+Epi	Δ*qseC* vs. Δ*qseC*+Epi	Δ*qseB* vs. Δ*qseB*+Epi
Outer membrane usher protein ycbS precursor	ycbS	1.19	NS	NS
Fimbrial chaperone yqiH precursor	yqiH	0.86	0.43	0.28
Fimbrial chaperone yfcS precursor	yfcS	0.96	0.46	NS
Putative outer membrane usher protein	htrE	1.47	-0.88	-0.42
Putative outer membrane usher protein	htrE	1.47	-0.88	-0.42
Putative chaperone protein EcpD	ecpD	1.22	NS	NS
Type-1 fimbrial protein, A chain precursor	fimA	0.76	-0.16	-0.32
Fimbrin-like protein fimI prec fimbrial Protein involved in type 1 pilus Biosynthesis	fimI	0.78	0.55	NS
F1C major fimbrial subunit precursor	focA	1.11	-0.29	-0.60
F1C periplasmic chaperone	focC	1.21	0.18	-0.16
F1C fimbrial usher	focD	1.58	-0.12	0.28
F1C minor fimbrial subunit F precursor	focF	1.27	0.77	0.41
Putative minor F1C fimbrial subunit Precursor	sfaD	1.59	0.09	NS
Putative F1C and S fimbrial switch Regulatory protein	sfaB	0.54	NS	-0.20
Putative F1C and S fimbrial switch Regulatory protein	sfaC	0.91	-0.55	0.25
PapA protein	papA_2	0.91	-0.16	0.50
PapC protein	papC_2	0.92	0.21	NS
PapE protein	papE_2	0.95	1.12	NS
PapH protein	papH_2	1.38	-0.36	-0.49
PapG protein	papG_2	0.75	-0.19	0.28
PapI protein	papI_2	1.23	0.59	0.38
PapJ protein	papJ_2	1.11	0.48	0.20
PapK protein	papK	0.58	-0.18	-0.14

NS (not significant)

Table 11. Fimbria and flagella related gene expression in AIEC strain static biofilm.

Gene function	Gene title	Fold change log2		
		146 vs 146+Epi	Δ*qseC* vs. Δ*qseC*+Epi	Δ*qseB* vs. Δ*qseB*+Epi
Putative fimbrial-like adhesin protein †	yadC	1.44	-0.33	0.43
Fimbrial-like protein yadN precursor †	yadN	1.22	0.11	0.08
Fimbrial-like protein yfcP precursor †	yfcP	1.39	0.19	0.43
Fimbrial-like protein yfcQ precursor †	yfcQ	1.45	NS	-0.07
Fimbrial-like protein yfcV precursor †	yfcV	1.31	-0.85	NS
Outer membrane usher protein ycbS Precursor †	ycbS	1.19	NS	NS
Outer membrane usher protein yehB precursor †	yehB	0.89	-0.17	0.40
Hypothetical protein †	yadK	0.84	-0.20	0.28
Hypothetical protein †	yadL	0.61	-0.55	-0.51
Hypothetical protein †	yfcR	1.46	0.31	0.16
Hypothetical protein †	yfcU	0.84	0.09	-0.62
Putative minor fimbrial subunit precursor †	c4210	1.13	0.27	0.62
Putative major fimbrial subunit precursor †	c4214	0.81	-2.50	-0.71
Flagellar protein flhE precursor §	flhE	1.09	-0.34	0.40
Flagellar basal body rod protein FlgF §	flgF	1.75	NS	-0.45
Flagellin §	fliC	1.45	NS	0.60
Flagellar assembly protein H §	fliH	1.52	-0.21	0.28
Transcriptional activator FlhC /// DNA-binding transcriptional dual regulator with FlhD §	flhC	0.94	-2.82	-2.00
Flagellar biosynthesis protein FliR§	fliR	1.11	0.40	-0.13

NS(not significant), †(fimbria synthesis genes), §(flagella genesis genes) according to DAVID database.

Iron transport genes

The expression levels of iron transport-related genes were markedly regulated by the *qseBC* operon in AIEC biofilms. Initially, treating UM146 biofilm with Epi showed that all of the genes involved in iron acquisition were up-regulated, having FC values higher than 0.5 (Tables 12a, 12b). The list included the siderophore gene (*ybiL*), enterobactins (*entE, entF*) and yersiniabactin FyuA (c2436); the presence of which are considered to enhance the pathogenicity of *E. coli* strains. Conversely large numbers of iron transport-related genes were down-regulated in the biofilms of Δ*qseC* and Δ*qseB* mutants. In the Δ*qseC* mutatant it was observed that more than 90% of the genes showed a decreased expression level, with *fecD* and *fhuB* genes with FC values of 0.58 and 0.25 respectively being the exception. Similarly Δ*qseB* contained five genes that were still active, including *entE*, *fepE*, *fepG*, *fhuB* and c5174 with FC values 1.14, 0.41, 0.38, 0.68 and 0.34 respectively (Table 12a). The yersiniabactin system had half the genes down-regulated and the other half had FC values near 0.5 in the Δ*qseC* mutatant. The Δ*qseB* mutant yielded six out of 16 genes that were down-regulated and only three had FC values superior to 0.5 (Table 12b).

Cellulose synthesis genes

Cellulose is one component of the biofilm matrix in *E. coli*. The microarray experiments detected three genes related to the production of this polysaccharide, including cellulose synthase catalytic subunit (*bcsA*), cyclic di-GMP-binding protein (*yhjN*) and cellulose synthase subunit (*yhjL*) (Table 12a). It was found that those genes remained up-regulated despite mutations present in the *qseBC* operon, showing in the

majority of the array comparisons FC values greater than 0.5. The parental strain UM146 array had FC values of 1.34, 1.08 and 1.92 for the *bcsA, yhjN* and *yhjL* genes respectively; while the Δ*qseC* and Δ*qseB* arrays had a slight decrease in FC values. The Δ*qseC* mutant showed dissimilar values of 0.41, 1.08 and 0.73 FC for the *bcsA, yhjN,* and *yhjL* genes, whereas Δ*qseB* had more continuous FC with 0.69, 0.72 and 0.79 values respectively for the same genes (Table 12a).

Table 12$_a$. Iron transport and cellulose production gene expression in AIEC biofilm.

Gene function	Gene title	Fold change log2		
		146 vs 146+Epi	Δ*qseC* vs. Δ*qseC*+Epi	Δ*qseB* vs. Δ*qseB*+Epi
Phosphopantetheinyltransferase component of enterobactin synthase multienzyme complex †	entD	1.20	NS	-0.36
Catecholate siderophore receptor Fiu †	ybiL	1.13	-0.62	NS
Enterobactin synthase subunit E †	entE	1.23	-0.51	1.14
Enterobactin synthase subunit F †	entF	0.91	NS	-0.07
Enterobactin/ferric enterobactin esterase †	Fes	0.75	-4.60	-3.16
Iron(III) dicitrate transport system permease protein fecD †	fecD	0.76	0.58	-0.13
Ferric enterobactin transport protein FepE †	fepE	1.37	-0.46	0.41
Iron-enterobactin transporter membrane protein†	fepD	1.19	NS	-0.56
Iron-enterobactin transporter permease †	fepG	1.30	-0.79	0.38
Ferrichrome outer membrane transporter †	fhuA	0.74	-0.32	NS
Outer membrane receptor FepA †	fepA	1.68	-1.28	-0.80
Iron-hydroxamate transporter permease subunit†	fhuB	1.59	0.25	0.68
Putative glucosyltransferase†	iroB	0.90	NS	-0.30
Outer membrane receptor FepA †	iroN	1.08	-1.28	-0.80
Ferric enterochelin esterase †	iroD	0.80	-0.13	-0.64
Putative iron-regulated outer membrane virulence protein †	c5174	1.36	-0.27	0.34
Cellulose synthase catalytic subunit §	bcsA	1.34	0.41	0.69
Cyclic di-GMP-binding protein §	yhjN	1.08	1.08	0.72
Cellulose synthase subunit BcsC §	yhjL	1.92	0.73	0.79

NS (not significant), † (iron transport genes), § (cellulose synthesis genes) according to DAVID database.

Table 12_b. Expression of genes involved in the yersiniabactin siderophore system in AIEC biofilm.

Gene function	Gene title	Fold change log2		
		146 vs 146+Epi	Δ*qseC* vs. Δ*qseC*+Epi	Δ*qseB* vs. Δ*qseB*+Epi
Prophage P4 integrase	c2418	0.85	-0.7	-0.43
Salicylate synthase Irp9	c2419	1.12	0.23	0.19
Putative cytoplasmic transmembrane Protein	c2420	1.59	-0.40	NS
Putative inner membrane ABC-transporter	c2422	0.90	-3.14	-0.20
Putative AraC type regulator	c2423	0.91	0.50	-0.62
Putative peptide synthetase	c2424	0.86	-0.12	NS
Putative peptide synthetase	c2426	1.24	-0.80	0.65
Putative peptide/polyketide synthetase Protein	c2427	1.12	0.27	0.25
Hypothetical protein	c2428	1.30	0.26	-0.16
Hypothetical protein	c2429	2.15	0.06	0.85
Hypothetical protein	c2430	1.38	0.23	0.25
Hypothetical protein	c2431	1.61	0.31	0.26
Putative salicyl-AMP ligase	c2434	1.45	-0.77	-0.26
Hypothetical protein	c2435	1.17	-0.33	0.18
Putative pesticin receptor precursor	c2436	1.45	-1.06	-0.16
Hypothetical protein	c2437	1.22	0.19	0.55

NS (not significant)

Invasion to HT29 cells in the presence of Epi and isogenic mutants.

The human adenocarcinoma epithelial cell line HT-29 has been used extensively by other researchers to evaluate the mechanisms by which AIEC invades host cells. The present study highlights the importance of Epi over the invasion procedure mediated by the *qseC* gene receptor (Figure 7). Initially it was demonstrated that mutating the *luxS*, *fimH* and *qseB* genes did not significantly decrease the intracellular bacterial numbers compared with the parental strain (UM146). On the other hand, the Δ*qseC* mutation decreased these numbers significantly by one log scale ($P < 0.05$). Furthermore, it was found that adding Epi at 50 μM significantly increased the amount of invading bacteria in the parental strain group. Nevertheless, adding this hormone to the Δ*fimH and* Δ*qseC* mutants tended to increase intracellular bacterial numbers but this increase was not significant (Figure 7). Patterns of these results are consistent with our previous motility assay results, demonstrating the importance of the AI-3/Epi/Ne QS over the AIEC invasion process.

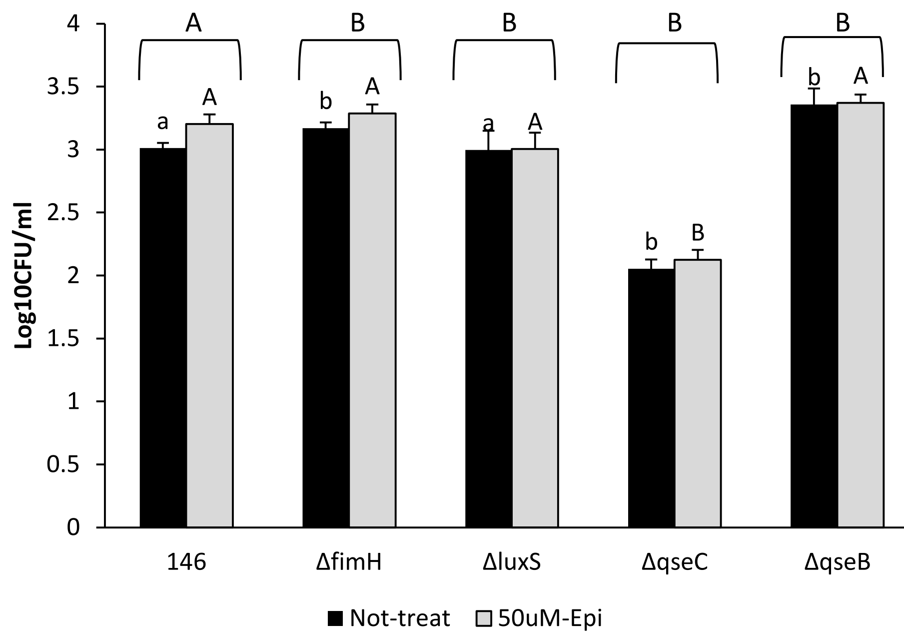


Figure 7. Invasion assay of cell line HT-29. Bacteria were counted after treatment with gentamycin (100µg/ml), and the data was log transformed. T-test was conducted comparing the wild type group (UM146) against the treatment and within the mutants. Subscripts with different letters are significant at P< 0.05.

DISCUSSION

Our microarray analysis revealed that a large number of genes involved in AIEC two-component systems were regulated by the *qseBC* operon mediated by the presence of the host hormone Epi. Genes involved include: the multidrug efflux system, nitrogen regulation protein, potassium transporting protein, transcriptional regulators and sensor proteins. Among these, the *baeR* and *baeS* genes conform to a two component system by which *E. coli* regulates several biological functions, including multidrug efflux systems, which confers *E. coli* resistance to the antibiotic novobiocin (Baranova and Nikaido, 2002). Other functions regulated by the *baeSR* system include protection against tannin compounds (Zoetendal *et al.,* 2008) and regulation of flagella synthesis (Nishino *et al.,* 2005). The latter study reported an up-regulation of flagella genes in a K-12 strain upon over expression of the *baeR* gene. Nevertheless, the mechanism of this regulation was not elucidated. Interestingly, we observed in our results that *baeR* was down regulated by the *qseC* mutation despite the presence of Epi.

Furthermore, the *torS* gene was also down regulated by the *qseC* mutation. This gene codes for a sensor kinase protein, TorS, and together with TorR form another two component system that apparently regulates expression of genes allowing *E. coli* to survive extreme pH conditions (Bordi *et al.,* 2003). Similarly, KdpD, a sensor protein involved in the potassium up-take (Epstein, 2003), was down regulated by the *qseC* mutation. However, no reports have indicated the role of these two component system over AIEC biofilms. This evidence demonstrates the regulatory impact of the adrenergic

receptor, *qseC*, and its response regulator, *qseB*, on several other two component systems in AIEC.

It is noteworthy that not all two-component system genes were affected by mutations in the *qseBC* operon. In particular the outer membrane porin protein C, *ompC*, remained up-regulated in all cases. This porin mediates changes in osmolarity during growth conditions, and it seems to be more active during biofilm formation (Pruss et al., 2006; Beloin et al., 2008). It has been established that *ompC* expression is regulated by the EnvZ/OmpR two-component system that modulates the colony formation in biofilm surfaces (Pruss et al., 2006), and is implicated in the *E. coli* establishment in the mammalian gut (Giraud et al., 2008). In AIEC strains, *ompC* plays an indirect role in the epithelial cells invasion process via the sigma factor RpoE (Rolhion et al., 2007). As AIEC can use this alternative pathway to invade the host, it may be possible to argue that AIEC could use the EnvZ/OmpR system to continue forming a biofilm in the absence of either *qseC* or *qseB* genes, but further studies should be conducted to confirm this hypothesis.

Fimbria and flagella synthesis are common factors studied during biofilm formation in *E. coli* (Beloin et al., 2008). However, addressing global gene expression during biofilm experiments varies greatly, making comparisions within studies extremely difficult, as previously mentioned. For instance, there are reports of using K-12 strains under poor growing conditions (Zhou et al., 2003; Ren et al., 2004; Beloin et al., 2004), of the O157:H7 strain forming biofilm over a glass surface with LB medium (Bansal et al., 2007), and using the CFT073 strain grown under human urine conditions (Hancock

and Klemm, 2007). Despite the controversy among studies, some similar patterns were observed in our results. Ren et al. (2004) found an up-regulation of genes involved in type 1 fimbria in K-12, and a similar situation was reported by Beloin et al. (2004) whereby the *fimA* and *fimI* genes were up-regulated during biofilm formation. In our case, we also found an up regulation of these two genes in the wild type strain (UM146) microarray. However, the *fimA* gene particularly was down regulated in the *qseC* mutant array comparison. In addition, we found that other fimbriae genes such as *foc, sfa* and *pap* remained active in the wild type microarray. These fimbriae genes are important for CFT073 infection of the urinary tract (Virkola et al., 1988; Holden et al., 2006), but their mechanism of action during infection remains unclear (Simms and Mobley, 2008).

Caution must be taken when drawing conclusions from this complex scenario. Some studies reported that many genes involved in the flagella-motility network can be inactive once they are in contact with a surface, whereas fimbriae genes can be found to be up-regulated (Ren et al., 2004; Beloin et al., 2004). Although these studies examined biofilms under poor growing conditions, they concentrated their efforts at 24 hours whereas our biofilm microarray study was performed at 12 hours and supplemented with high Epi concentrations of 100 μM. In this sense, we did not find a similar pattern as that reported by Bansal et al. (2007) where the addition of Epi to biofilms down-regulated several genes involved in the flagella-motility network, but not all these genes were down-regulated. Perhaps the discrepancy between results can be explained by the differences in the biofilm media used (LB vs. minimal media), different strains (O157:H7 vs. AIEC), and the type of surface used in biofilm experiments (glass vs. plastic).

Nonetheless, overall it was found that mutations in the *qseBC* operon drastically affected flagella-motility gene expression and were dependent on the presence of host hormones like Epi. This concurs with investigations conducted on O157:H7 strains (Kendall et al., 2007; Clarke et al., 2007).

Bacteria essentially use iron to grow and *E. coli* has developed powerful systems to sequester iron from its host through the expression of siderophores (Raymond et al., 2003). Uropathogenic *E. coli* like CFT073 contains genes encoding multiple siderophores in its genome. For instance, enterobactin steals iron from the host while evading activation of the immune system via the *iroA* cluster genes. This cluster contains several genes including *iroB, C, D, E* and *N* which are responsible for the modification and transport of iron into the CFT073 cytoplasm (Fischbach et al., 2006). Another siderophore, the yersiniabactin, helps to enhance CFT073 biofilm formation under iron-depleted conditions, such as in human urine via activation of the outer membrane protein FyuA (Hancock et al., 2008). Other pathogenic *E. coli* like O157:H7 contain siderophores for iron uptake from their environment (Torres and Payne, 1997), and gene expression of many iron-transport systems in this bacterium is regulated by the AI-3/Epi/Ne QS system (Hughes and Sperandio, 2008). In this study, we found that an AIEC strain contains both siderophore-related genes - enterobactin and yersiniabactin. Both are under the control of the *qseBC* operon inasmuch as many iron-transport related genes such as *iroN, iroD* and *fyuA* (c2436) were down-regulated either in *qseC* or *qseB* mutations.These findings coincide with the information reported for O157:H7 by Bansal et al. (2007) and Hughes

and Sperandio (2008). However, the overall effect of the two siderophores on biofilm and pathogenesis of AIEC under in-vivo conditions still needs to be investigated.

Cellulose is one of the polysaccharides comprising the matrix of biofilms in bacteria. Although its overall function remains somewhat unknown, it is believed it confers protection against precarious conditions and is also related to biofilm formation in *E. coli* (Beloin et al., 2008). Cellulose synthesis requires the operon *bcsABZ* whereby the *bcsA* and *bcsB* genes are part of the cellulose synthesis complex (Romling, 2005). In the present study, AIEC biofilm microarray analysis showed that three genes involved in the synthesis of cellulose (*bcsA, yhjN,* yhjL), were not under the regulation of the AI-3/Epi/Ne QS system, since they remained active despite the *qseB* and *qseC* mutations and the Epi treatment. These results were not a surprise because the activity of *bcsA* and *bcsB* are preferentially under control of a small molecule called cyclic-di-GMP (c-di-GMP) (Beloin et al., 2008; Weber et al., 2006; Da Re and Ghigo, 2006). The biological pathways involving the synthesis of c-di-GMP and its regulatory effects on gene expression in *E. coli* is wide and complex (Weber et al., 2006; Jonas et al., 2008). Additionally, other biological functions linked to c-di-GMP include motility and curli fimbria (Weber et al., 2006; Sim et al., 2004). Recently it was determined that cellulose in conjunction with curli are necessary to promote biofilm formation and host colonization in pathogenic O157:H7 (Saldaña et al., 2009). Unfortunately, investigations involving cellulose production in AIEC strains biofilms are unknown. Thus it could be speculated that under our experimental conditions the high biofilm formation in the Δ*qseC* and Δ*qseB* mutants may be explained because of the presence of genes involved in

cellulose synthesis, they remained up-regulated, and their activity could be controlled by the c-di-GMP molecule and not by the AI-3/Epi/Ne QS system. However, further research is needed to corroborate this hypothesis.

It has been established that invasion to epithelial cells is one of the hallmark characteristics of AIEC strains based on previous investigations (Martin et al., 2004). Therefore we were interested to investigate the effect of Epi over the AIEC invasion process as well as determining if mutations present in genes involved in QS and biofilm formation can affect this process. Initially, we did not find a Δ*fimH* mutation effect on the invasion to HT-29, which is surprising because previous reports indicate a decrease in the number of this type of pathogenic *E. coli* once mutations are present in the *fimH* gene (Carvalho et al., 2007). However, the same pattern was repeated in our motility assays, indicating that other factors were involved in AIEC invasion. In this sense, we found that mutating the *qseC* gene drastically reduced the AIEC UM146 invasion of HT-29 cells as well as motility, and microarray analysis showed that a large set of genes involved in flagella synthesis and fimbria were down-regulated. It has also been proven that flagella proteins are needed by AIEC to invade to epithelial cells (Barnich et al., 2003; Claret et al., 2007), which helps to explain the fact that the *qseC* mutant diminished the invasive bacteria in our study.

It is interesting to observe that adding the stress hormone Epi increased the amount of intracellular bacteria in the parental strain (UM146) but not in the *qseC* mutant. This indicates that AIEC UM146 uses this adrenergic receptor to sense the hormone, and thus modulates its invasion of epithelial cells via the flagella and fimbria

network. This information coincides with investigations conducted in O157:H7, prooving the regulatory effect of cathecholamines on the flagella network (Sperandio et al., 2002; Clarke et al., 2006). Nevertheless, despite invasion and motility being decreased in the *qseC* mutant, it was still capable of invading HT-29 to a lesser degree. This can be explained by some genes which are involved in flagella and fimbria factors remaining active despite mutations in the *qseBC* operon and treatment with Epi based on our microarray analysis. This situation has been observed in other studies where of flagella and fimbria gene expression were also variable (Kendall et al., 2007; Bansal et al., 2007), meaning that the invasion of epithelial cells is more complex and involves several pathways that occur in a similar way to biofilm formation (Pruss et al., 2006). For instance, in the absence of a functional *qseBC* operon, AIEC could be using a osmolarity regulated protein like *ompC* to continue invading epithelial cells (Rolhion et al., 2007).

CONCLUSIONS

In summary, this study highlights the gene expression profiles of AIEC biofilms supplemented with epinephrine and the effect of mutations in the *qseBC* operon which encodes the adrenergic receptor QseC. This allows bacteria to communicate with its host in a process generally known as QS involving specifically the AI-3/Epi/Ne system. AIEC strains can use this system to regulate the expression of a large set of genes involved in a myriad of biological functions implicated in biofilm formation, including flagella, fimbria synthesis and iron transport as well as playing a role in the invasion to epithelial cells. But this regulation is not absolute because many genes employed in flagella and fimbria functions remained active despite mutations in the *qseC* and *qseB* genes. The AIEC biofilm, just as those formed by other *E. coli* strains, may have alternative pathways that regulate the expression of genes involved in biofilm. Based on our results the proposed pathways can include the operon EnvZ/OmpR and the cyclic-di-GMP molecule. The former is a two-component system involved in the regulation of porins like OmpC, permitting *E. coli* to sense osmolar changes in the growing medium and establishing microcolonies on a surface. The latter is a small second messenger molecule involved in regulating the production of cellulose which is part of the biofilm matrix. Nonetheless, it is important to determine in future studies whether these two pathways are dependent on other signals produced by the host and its mechanisms involved in AIEC pathogenesis.

OVERALL CONCLUSIONS

The ability of *E. coli* to form biofilms differs among strains and can be highly influenced by the conditions in which the biofilm studies are conducted (Reisner et al., 2006). In addition to the culture conditions, variations in biofilm formation between *E. coli* strains depend on the presence of ancillary factors that enhance or decrease biofilm establishment. These factors include the presence of flagella, fimbria, conjugative plasmids and production of polysaccharides like cellulose (Beloin et al., 2008). The first experiment in this thesis (manuscript I) was conducted as a survey of diverse *E. coli* strains and attempted to correlate their biofilm formation with the presence or absence of adhesins and QS-related genes. The aim of this experiment was to explain whether the formation of biofilm could be affected by different factors, including medium growth conditions, presence of adhesins and QS-related genes, in particular AI-2 genes. Data from this investigation illustrated a great deal of variation in the degree of biofilm formation by environmental *E. coli* under in-vitro conditions. As with other studies, it was found that media conditions had a marked impact on biofilm development (Reisner et al., 2006; Skyberg et al., 2007; Yang et al., 2006). In general this report found that minimal media (M9) facilitated greater biofilm formation compared with M9 supplemented either with CRF or PWF (Tables 3 and 4). However for some strains, adding both components into M9 increased the formation of biofilms. There are several plausible hypotheses to explain this variation, for instance there is a lack of knowledge about the composition of CRF and PWF. CRF is known to be a source of volatile fatty acids comprised of mainly acetic, propionic and butyric acid (Calberry et al., 2003). It has

also been reported that CRF contains acyl homoserine lactones (AHL) (Erickson et al., 2002). Despite this information, many elements of CRF and PWF are still unknown, leading to speculation that at a certain point *E. coli* might use some of these substances to either increase or decrease biofilm formation.

Autotransporter protein adhesins also have an impact on biofilm formation (Van Houdt and Michiels, 2005). The role of AIDA and Ag43 in biofilm formation in pathogenic *E. coli* strains is well documented (Sherlock et al., 2004; Ravi et al., 2007; Ulett et al., 2007b). Ag43 promotes biofilm formation by enhancing microcolony formation (Van Houdt and Michiels, 2005). AIDA can also enhance the formation of biofilms in pathogenic and non-pathogenic *E. coli* under in-vitro conditions (Sherlock et al., 2004; Ravi et al., 2007). In most cases, studies conducted on AIDA and Ag43 have focused on UPEC and K12 strains. In our survey, we included broad non-domesticated *E. coli* strains which vary from K-12 and UPEC (Table 5). Despite our multivariance analysis indicating AIDA and Ag43 did not have a significant impact on biofilm formation (Figure 1), it does not mean that these adhesins are not involved in biofilm formation. It is safe to argue that these adhesins play a role during the initial stages of biofilm development when the bacteria are first attaching to the surface.

Surprisingly, our results revealed that the majority of wild type *E. coli* examined in this survey did not harbor many of the genes involved in the AI-2 network, particularly the *mqsR* gene (Table 6). Noteworthy, *mqsR* was present in *E. coli* strains belonging to the phylogenetic group A where K-12 is grouped (Clermont et al., 2000). Statistical analysis indicated that our strains formed a biofilm despite the presence or absence of

these genes. These findings contrast with those reported for K-12 where mutations in the *luxS* and *mqsR* genes decreased AI-2 synthesis, which adversely affected K-12 biofilm development (Wood and Bentley, 2007). Based on our data, we hypothesized that another system may be regulating biofilm formation. It was speculated that our wild type strains used the AI-3 system to regulate biofilm formation instead. Notably, many strains came from gastrointestinal origins like cattle feces and human gut tissue. Perhaps under gut conditions *E. coli* requires a specific QS system, like the AI-3 system, to recognize and colonize the host. The AI-2 system in our studies, as described in K-12 is clearly not as widespread as one might think. Many genes purported to be important in K-12 were not present in several of our isolates even though these isolates formed biofilm. Particularly, the human strains lacked many AI-2 related genes but were still capable of forming biofilms.

Given that genes encoding several adhesions and AI-2 QS components were missing, particularly in the human *E. coli* strains (Tables 5 and 6) and those strains were capable of forming a biofilm, we hypothesized that other QS systems may be involved in the biofilm process. To further understand the role of QS in biofilm in adherence and AIEC, the second study (manuscript II) was conducted to determine if the AI-2 or AI-3 system regulates flagella-motility or other factors that could affect biofilm formation in AIEC *E. coli*. To evaluate the potential role of *luxS*, which was present in AIEC, we created a *luxS* mutant. We observed that a *luxS* mutation had no effect on biofilm formation, but when Epi was added to the medium at either 50 µM or 100 µM, there was an increase in biofilm development (Figure 3). Based on in-silico analysis, it was

proposed that a bacterium should have a transport system for the AI-2 molecule for QS to be functional; otherwise the presence of the *luxS* gene may be limited to methionine metabolism (Rezzonico and Duffy, 2008). Initial studies in O157:H7 suggested that *luxS* was involved with AI-3 synthesis because a *luxS* mutant exhibited reduced the AI-3 production (Sperandio et al., 2003). However, it was later determined that the role of AI-2 has yet to be fully determined as *luxS* was associated with sulphur amino acid metabolism in this bacterium (Walters et al., 2006). In addition, AIEC motility was unaffected by mutating *luxS* but was increased by adding Epi (Figure 5). Thus it appears that human pathogenic AIEC contains the gene responsible for producing the AI-2 molecule (*luxS*) but its effect did not appear to be involved in bacterium motility and biofilm formation. Thus, other QS systems may be more important in AIEC, such as the AI-3/Epi/Ne system.

In *E. coli* O157:H7, the AI-3 molecule is involved in the activation of LEE and flagellar genes (Clarke and Sperandio, 2005b; Pacheco and Sperandio, 2009). Although the structure of the AI-3 molecule is not known, it is probably an aromatic compound with structural similarities to Epi and Ne as these hormones appear to bind the same bacterial receptor. The gene product of *qseBC* is a two-component signal transduction system. Once the sensor kinase QseC senses AI-3/Epi/Ne, it becomes autophosphorylated, and the signal is then transduced to the regulator QseB, enabling it to activate transcription of the master flagella regulon, *flhDC* (Clarke et al., 2006; Hughes et al., 2009). In *E. coli* O157:H7, LEE is also regulated by AI-3, and Epi/Ne induces the transcription of the LEE locus. If *qseC* is mutated LEE may not be transcribed in the

presence of Epi/Ne (Hughes et al., 2009). However, AIEC UM 146 does not have a LEE, and the best described adherence factor is *fimH*, which encodes the terminal subunit of the type I pilus structure (Sepehri et al., 2009). It is known that FimH has a structure which is conserved among AIEC, as well as some urinary tract isolates (CFT073) and avian pathogenic *E. coli* (APEC O1) (Sepehri et al., 2009). There is a host-specific attachment by FimH to CEACAM6 in humans and when fimH is mutated, AIEC will not adhere to and invade epithelial cells (Glasser and Darfeuille-Michaud, 2008). We demonstrated that *fimH* mutations do not result in a reduction in biofilm formation even in the presence of Epi. To investigate the potential relationship between Epi in the wild type and *luxS* and *fimH* mutants, we investigated its effects on the flagella regulon (Figure 4). We demonstrated that *qseC* and *flhD* were up-regulated in the presence of Epi in a dose-dependent manner (Figure 4). This response was independent of the luxS or fimH mutations. These observations are in agreement with Pacheco and Sperandio (2009).

The flagella regulon influences motility and the luxS and fimH mutations did not affect motility in the presence of Epi compared to the wild type controls. However, when we mutated qseB or *qseC* there was no increase motility in the presence of Epi (Figure 5). Hughes et al. (2009) mutated qseB and *qseC* in *E. coli* O157:H7 and measured motility in the presence of AI-3. They found no effect of AI-3 in the qseB mutant but a reduction of motility in the *qseC* mutant. Clarke et al. (2006) found no effect of Epi on motility in *qseC* mutants of *E. coli* O157:H7. It is clear from our results that the AIEC strain UM146 responds to Epi which affects motility.

The Epi/Ne/AI-3 system of autoinducer signalling is likely to be fairly widespread in enteric bacteria (Pacheco and Sperandio, 2009). In *E. coli*, the greatest genetic similarity probably lies within AIEC, avian pathogenic *E. coli* and urinary tract *E. coli*. The phylogenetic relationships between fimH, luxS, qseB and *qseC* indicate a high level of similarity, suggesting a common evolutionary origin (Figure 2). The urinary tract pathogen *E. coli* CFT073 is known to form of biofilm in the urinary tract (Allsopp et al., 2010) and avian pathogenic *E. coli* is highly adapted to biofilm formation (Skyberg et al., 2007). In this report, we found that mutating *qseC* and *qseB* did not decrease biofilm formation by our AIEC strain. In fact the opposite situation happened; the mutants were capable of producing higher levels of biofilm than the parental strain (Figure 6). More intriguing was the finding that addition of Epi did not enhance AIEC biofilm formation. In this situation, we suspect that more than one biological pathway may be involved in the AIEC biofilm phenomenon (Pruss et al., 2006), which may take over in the absence of the *qseBC* operon.

The aim of the last study (manuscript III) was to obtain a global gene expression profile of the wild type AIEC and the *qseB* and *qseC* mutants when growing as a biofilm. Initially it was found that a large number of genes involved in the AIEC two-component systems could be regulated by the *qseBC* operon mediated by the presence of the host hormone Epi. Genes involved include the multidrug efflux system, a nitrogen regulation protein, a potassium transport protein, transcriptional regulators and sensor proteins (Table 9). Among these, the *baeR* and *baeS* genes encode a two-component system which regulates several biological functions including the multidrug efflux system,

confering resistance to the antibiotic novobiocin (Baranova and Nikaido, 2002) and protection against tannin compounds (Zoetendal et al., 2008), as well as regulation of flagella synthesis (Nishino et al., 2005). Other alleles affected by mutations in the *qseBC* operon independent of Epi include the *torS* and *kdpD* genes. The former gene codes for a sensor kinase protein, TorS, which together with TorR forms another two-component system that enables *E. coli* to survive extreme pH conditions (Bordi et al., 2003). The second gene encodes KdpD, a sensor protein involved in potassium uptake (Epstein, 2003). Although no reports have indicated the role of two-component systems in AIEC biofilms, our findings suggest that the adrenergic receptor QseC and its response regulator QseB regulate expression of several other AIEC two-component systems.

Importantly, not all two-component system genes were affected by mutations in the *qseBC* operon. For example, the gene encoding the outer membrane porin OmpC, remained up-regulated in all cases (Table 9). Expression of this porin fluctuates in response to changes in osmolarity, and OmpC seems to be more active during biofilm formation (Pruss et al., 2006; Beloin et al., 2008). It has been established that *ompC* transcription is regulated by the EnvZ/OmpR two-component system which also modulates colony biofilm formation (Pruss et al., 2006) and is implicated in the establishment of *E. coli* in the mammalian gut (Giraud et al., 2008). In AIEC strains, OmpC plays an indirect role on the invasion of epithelial cells via the sigma factor RpoE (Rolhion et al., 2007). As AIEC can employ this alternative pathway to invade the host, one could argue that AIEC uses the EnvZ/OmpR system to continue forming biofilms in

the absence of either the *qseC* and *qseB* genes. Further studies should be conducted to confirm this hypothesis.

Fimbria and flagella are factors that play a role in biofilm formation in *E. coli* (Beloin et al., 2008). Studies addressing global gene expression in biofilms vary greatly, making it difficult to compare fimbria and flagella gene expression in these studies. There are reports investigating K-12 strains grown under low nutrient conditions (Ren et al., 2004; Beloin et al., 2004), strain O157:H7 grown in LB medium (Bansal et al., 2007), and strain CFT073 grown under human urine conditions (Hancock and Klemm, 2007). Despite the differences observed among these studies, similar patterns can be found in our results. Ren et al. (2004) found an up-regulation of genes involved in type I fimbria in K-12. Similar results were reported by Beloin et al. (2004) where the *fimA* and *fimI* genes were up-regulated during biofilm formation. In our study, we also found an up-regulation of these two genes in the wild type strain (UM146) microarray, but not in the *qseC* mutant array. In addition, we discovered that other fimbriae genes such as foc, sfa and pap remained active in the wild type microarray (Table 10). These fimbriae genes are important for CFT073 infection in the urinary tract (Virkola et al., 1988; Holden et al., 2006), but their exact role in infection remains unclear (Simms and Mobley, 2008).

Studies reported that many genes involved in the flagella-motility network are repressed once they are in contact with a surface, whereas fimbriae genes are often up-regulated (Ren et al., 2004; Beloin et al., 2004). We did not find a similar pattern with the data reported by Bansal et al. (2007) in which the addition of Epi to biofilms down-regulated several genes involved in the flagella-motility network, and fimbriae. Perhaps

the discrepancy between results can be explained by the differences in the media (LB vs. minimal media), the strains examined (O157:H7 vs. AIEC), and the type of surface used in the biofilm experiments (glass vs. plastic). Nonetheless it was found that the mutations in the *qseBC* operon drastically affected flagella-motility gene expression and expression was dependent on the presence of host hormones like Epi. This is in agreement with investigations conducted on O157:H7 strains (Kendall et al., 2007; Clarke et al., 2007).

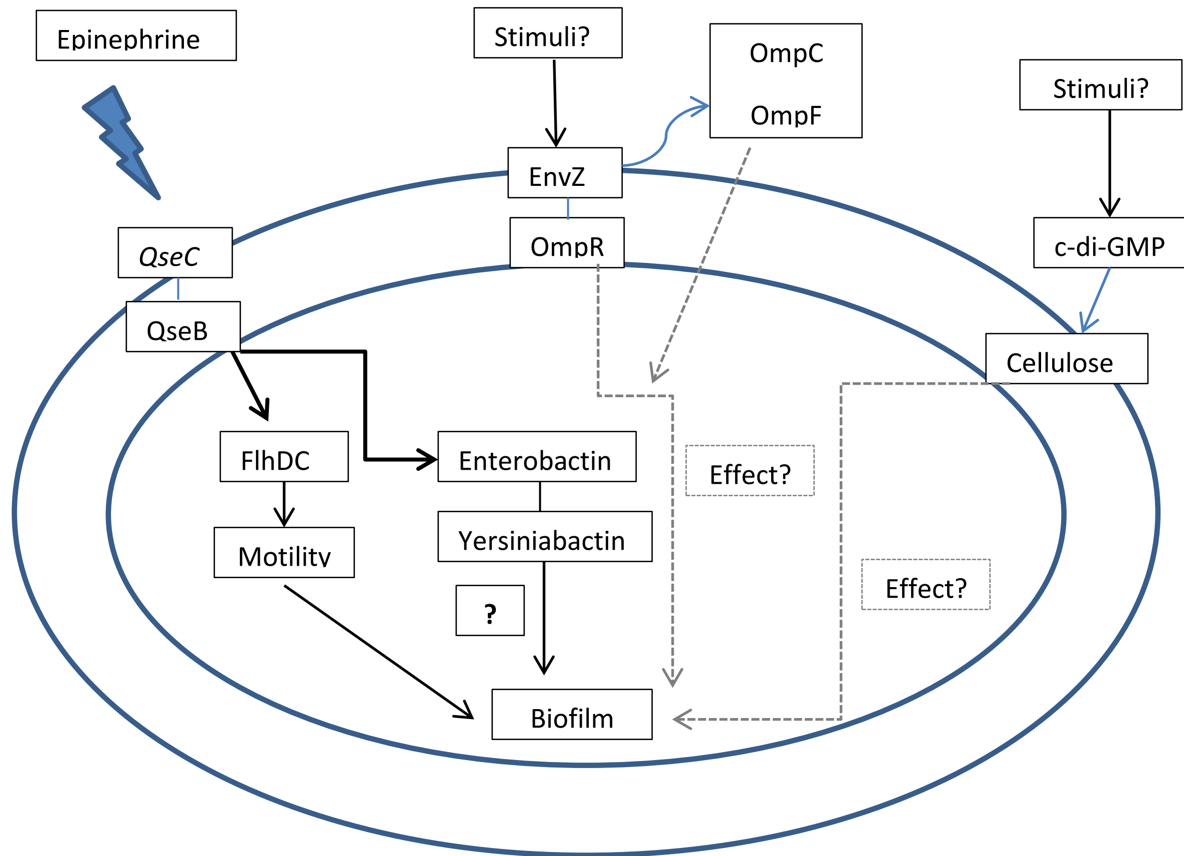
E. coli has compounds known as siderophores that enable the bacteria to sequester iron from the host (Raymond et al., 2003). Uropathogenic *E. coli* like CFT073 contain multiple genes encoding siderophores. For instance, enterobactin steals iron from the host while evading the activation of the immune system via the *iroA* cluster genes. This cluster contains several genes including *iroB, C, D, E* and *N* which code for proteins responsible for the modification and transport of iron into the CFT073 cytoplasm (Fischbach et al., 2006). The siderophore yersiniabactin enhances CFT073 biofilm formation under iron-depleted conditions, like those found in human urine, via activation of the outer membrane protein FyuA (Hancock et al., 2008). Other pathogenic *E. coli*, like O157:H7, contain siderophores to take up iron from their environment (Torres and Payne, 1997); gene expression of many iron-transport systems in this bacterium is under-regulation of the AI-3/Epi/Ne QS system (Hughes and Sperandio, 2008). In this study, we found that the AIEC strain contains genes for the siderophores enterobactin and yersiniabactin (Tables 12a and 12b). Both genes were under the control of the *qseBC* operon inasmuch as many iron-transport-related genes such as *iroN, iroD* and *fyuA* (c2436) were down-regulated either in the *qseC* or *qseB* mutants. This coincides with the

information reported in O157:H7 by Bansal et al. (2007) and Hughes and Sperandio (2008). However, the overall effect of the two siderophores on biofilm formation and the pathogenesis of AIEC under in-vivo conditions remains to be investigated.

Cellulose is one of the polysaccharides comprising the matrix of biofilms in bacteria. Although its overall function remains unknown, cellulose may confer protection against precarious conditions and is important for biofilm formation in *E. coli* (Beloin et al., 2008). Cellulose synthesis requires the operon *bcsABZ* whereby the *bcsA* and *bcsB* genes are part of the cellulose biosynthesis complex (Romling, 2005). In manuscript III, the AIEC biofilm microarray analysis showed that three genes involved in the synthesis of cellulose (*bcsA, yhjN, yhjL*) were not under the regulation of the AI-3/Epi/Ne QS system (Table 12a), as they remained active despite the presence of the *qseB* and *qseC* mutations and the Epi treatment. This result is not surprising because the expression of *bcsA* and *bcsB* is under control of a small molecule called cyclic-di-GMP (c-di-GMP) (Beloin et al., 2008; Weber et al., 2006; Da Re and Ghigo, 2006). The biological pathways involving the synthesis of c-di-GMP and its regulatory effects on gene expression in *E. coli* are wide and complex (Weber et al., 2006; Jonas et al., 2008). Other biological functions linked to c-di-GMP include motility and curli fimbria (Weber et al., 2006; Sim et al., 2004). Recently it was determined that cellulose in conjunction with curli fimbria are necessary to promote biofilm formation and host colonization in pathogenic O157:H7 (Saldaña et al., 2009). However, the role of cellulose production in AIEC biofilms is unknown. Thus it could be speculated that under our experimental conditions, enhanced biofilm formation in the Δ*qseC* and Δ*qseB* mutants may be due to

increased expression of genes involved in cellulose synthesis, the activity of which is controlled by the c-di-GMP molecule, not the AI-3/Epi/Ne QS system. Further research is needed to corroborate this hypothesis.

Figure 8. The schematics of biofilm formation in AIEC UM146. Solid arrows indicate the main pathways involved in the biofilm process. Gray dashed arrows indicates putative pathways involved in biofilm formation.



Invasion of a host tissue and cell lines is one of the remarkable characteristics of AIEC strains (Martin et al., 2004). We were interested in the effects of supplementing Epi in a set of mutants constructed in AIEC that could affect the invasion process (Figure 7). Surprisingly, we discovered no effect of the *fimH* mutation on the invasion of HT-29, which contrasts with previous reports (Carvalho et al., 2007). The same pattern was observed in our motility assays (Figure 5), indicating that other factors must be involved in these processes in AIEC. Notably we found that a mutation in the *qseC* gene drastically reduced the intracellular numbers in HT-29. Motility was severely affected by this mutation and the microarray analysis indicated that a large set of genes, which included many flagellra and fimbrial genes, were down-regulated (Table 11). This may explain the reduced invasion capacity exhibited by the *qseC* mutant.

Interestingly, adding Epi increased the intracellular bacterial numbers in the parental strain (UM146) but not in the *qseC* mutant. These findings indicate that AIEC UM146 uses this adrenergic receptor to sense the hormone and thus modulate its invasion of epithelial cells via the flagella and fimbria network. This coincides with reports conducted on O157:H7 (Sperandio et al., 2002; Clarke et al., 2006). However, in spite of the reduced invasion and motility by the *qseC* mutant, this strain was capable of invading HT-29 (Figure 7). This may be due to flagellar and fimbrial genes remaining active despite mutations in the *qseBC* operon and the treatment with Epi. As observed in other studies, flagella and fimbria gene expression was also variable (Kendall et al., 2007; Bansal et al., 2007), indicating that invasion of epithelial cells is more complex and

involves several pathways, similar to what was found for biofilm formation (Pruss et al., 2006).

In summary, the AIEC can use the AI-3/Epi/Ne system to regulate the expression of large set of genes, which are involved in a myriad of biological functions implicated in biofilm formation and invasion of epithelial cells which include flagella, fimbria synthesis and iron transport. However, regulation of gene expression during biofilm establishment was not entirely governed by the AI-3/Epi/Ne system, because many genes employed in the flagella and fimbria factors remained active despite mutations in *qseC* and *qseB*.

The AI-3/Epi/Ne system in AIEC generally operates in a manner similar to that in O157:H7. However, there are differences between those strains, for example AIEC does not have the pathogenicity island LEE, plus AIEC is an invasive strain while O157:H7 is not. In this thesis the AI-3/Epi/Ne system worked similar to what has been reported for O157:H7 for certain factors like the *qseBC*, *flhDC* operons and motility. But for others like *qseB* and invasion, distinct differences were observed. The AIEC, as with other *E. coli* strains, appears to have alternative pathways that regulate the expression of many genes involved in biofilm formation and invasion. In our microarray experiments, we reported that genes encoding the porin OmpC and cellulose remained active despite mutations in the *qseC* and *qseB* genes. Therefore, based on our results, these pathways may include the two-component system EnvZ/OmpR and the cyclic-di-GMP molecule.

FURTHER DIRECTIONS

1. In O157:H7 the link between AI-3/Epi/Ne system and virulence factors have been elucidated to a large extent. However, in pathogenic AIEC strains, other virulence factors that influence the invasion process, remains to be discovered.

2. AIEC contains two known QS systems (AI-2 and AI-3/Epi/Ne). However, the presence of other QS systems has not been discovered in AIEC. There is evidence of a third QS system in O157:H7 and K-12 strains called SdiA, but its role on the AIEC biofilm formation and invasion have not been elucidated.

3. We evaluated one stress hormone during this thesis (Epi), however other hormones like cortisol are present in the host which might have an impact on the AIEC pathogenesis, and until now it remains unknown the virulence factors that this particular hormone can influence in AIEC.

4. There seems to be a connection between stress and the catecholamines hormones in humans. Nevertheless, in IBD patients particularly CD, the intestinal hormone levels, in particular cortisol, should be quantified preferentially between extreme weather conditions like winter versus summer to make a correlation between stress and the incidence of AIEC.

5. In O157:H7 the link between QseC/B and virulence is the pathogenicity island LEE. In AIEC, determining which other virulence genes are regulated by QseC/B still needs to be stablished. Future research should elucidate the link between QseC/B and virulence factors that regulate invasion and survival of AIEC in macrophages and other eukaryotic cells.

LIST OF REFERENCES

Aas, J. A., B. J. Paster, L. N. Stokes, I. Olsen, and F. E. Dewhirst. 2005. Defining the normal bacterial flora of the oral cavity. J. Clin. Microbiol. 43: 5721-5732.

Agladze, K., X. Wang, and T. Romeo. 2005. Spatial periodicity of *Escherichia coli* k-12 biofilm microstructure initiates during a reversible, polar attachment phase of development and requires the polysaccharide adhesin pga. J. Bacteriol. 187: 8237-8246.

Allsopp, L. P., M. Totsika, J. J. Tree, G. C. Ulett, A. N. Mabbett,T. J. Wells, B. Kobe, S. A. Beatson, and M. A. Schembri. 2010. UpaH is a newly identified autotransporter protein that contributes to biofilm formation and bladder colonization by uropathogenic *Escherichia coli* CFT073. Infect. Immun. 78: 1659-1669.

Anderson, G. G., C. C. Goller, S. Justice, S. J. Hultgren, and P. C. Seed. 2010. Polysaccharide capsule and sialic acid-mediated regulation promote biofilm-like intracellular bacterial communities during cystitis. Infect. Immun. 78: 963-975.

Anonymous. 1997. Applied Biosystems Prism 7700 Sequence Detection System: user bulletin #2. Perkin-Elmer Corp., Norwalk, Conn.

Bahrani-Mougeot, F. K., E. L. Buckles,C. V. Lockatell, J. R. Hebel, D. E. Johnson,C. M. Tang and M. S. Donnenberg. 2002. Type 1 fimbriae and extracellular polysaccharides are preeminent uropathogenic *Escherichia coli* virulence determinants in the murine urinary tract. Mol. Microbiol. 45: 1079-1093.

Balsalobre, C., J. Morschhauser, J. Jass, J. Hacker, and B. E. Uhlin. 2003. transcriptional analysis of the sfa determinant revealing multiple mrna processing events in the biogenesis of S fimbriae in pathogenic *Escherichia coli*. J. Bacteriol. 185: 620-629.

Bansal, T., D. Englert, J. Lee, M. Hegde, T. K. Wood, and A. Jayaraman. 2007. Differential effects of epinephrine, norepinephrine, and indole on *Escherichia coli* O157:H7 chemotaxis, colonization, and gene expression. Infect. Immun. 75: 4597-4607.

Baranova, N., and H. Nikaido. 2002. The BaeSR two-component regulatory system activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and deoxycholate. J. Bacteriol. 184: 4168-4176.

Barnich, N., J. Boudeau, L. Claret, and A. Darfeuille-Michaud. 2003. Regulatory and functional co-operation of flagella and type 1 pili in adhesive and invasive abilities of AIEC strain LF82 isolated from a patient with Crohn's disease. Mol. Microbiol. 48: 781-794.

Barnich, N., F. A. Carvalho, A. L. Glasser, C. Darcha, P. Jantscheff, M. Allez, H. Peeters, G. Bommelaer, P. Desreumaux, J. F. Colombel, and A. Darfeuille-Michaud. 2007. CEACAM6 acts as a receptor for adherent-invasive *E. coli,* supporting ileal mucosa colonization in Crohn disease. J. Clin. Invest. 117: 1566-1574.

Barnich, N., and A. Darfeuille-Michaud. 2007. Role of bacteria in the etiopathogenesis of inflammatory bowel disease. World J. Gastroenterol. 13: 5571-5576.

Bassler, B. L., and R. Losick. 2006. Bacterially Speaking. Cell 125: 237-246.

Baumgart, M., B. Dogan, M. Rishniw, G. Weitzman, B. Bosworth, R. Yantiss, R. H. Orsi, M. Wiedmann, P. McDonough, S. G. Kim, D. Berg, Y. Schukken, E. Scherl, and K. W. Simpson. 2007. Culture independent analysis of ileal mucosa reveals a selective increase in invasive *Escherichia coli* of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum. ISME J. 1: 403-418.

Beloin, C., J. Valle, P. Latour-Lambert, P. Faure, M. Kzreminski, D. Balestrino, J. A. J. Haagensen, S. Molin,G. Prensier, B. Arbeille, and J. Ghigo. 2004. Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. Mol. Microbiol. 51: 659-674.

Beloin, C., A. Roux, and J.M. Ghigo. 2008. Escherichia coli biofilms. Pages 249-289 in: Bacterial biofilms current topics in microbiology and immunology. T. Romeo .ed. Springer-Verlag Berlin Heidelberg.

Blazejczyk, M., M. Miron, and R. Nadon. 2007. FlexArray: A statistical data analysis software for gene expression microarrays. GenomeQuebec, Montreal, Canada. Genome Quebec, Montreal, Canada [http://genomequebec.mcgill.ca/FlexArray/].

Bokranz, W. 2005. Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. J. Med. Microbiol. 54: 1171-1182.

Bolstad, B. M., R. A. Irizarry, M. Åstrand, and T. P. Speed. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19: 185-193.

Bonen, D. K., Y. Ogura, D. L. Nicolae, N. Inohara, L. Saab, T. Tanabe, F. F. Chen, S. J. Foster, R. H. Duerr, S. R. Brant, J. H. Cho, and G. Nuñez. 2003. Crohn's disease-

associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan. Gastroenterology 124: 140-146.

Bordi, C., L. Théraulaz, V. Méjean, and C. Jourlin-Castelli. 2003. Anticipating an alkaline stress through the Tor phosphorelay system in *Escherichia coli.* Mol. Microbiol. 48: 211-223.

Boudeau, J., N. Barnich, and A. Darfeuille-Michaud. 2001. Type 1 pili-mediated adherence of *Escherichia coli* strain LF82 isolated from Crohn's disease is involved in bacterial invasion of intestinal epithelial cells. Mol. Microbiol. 39: 1272-1284.

Callaway, T. R., M. A. Carr, T. S. Edrington, R. C. Anderson, and D. J. Nisbet. 2009. Diet, *Escherichia coli* O157:H7, and cattle: a review after 10 Years. Curr. Issues Mol. Biol. 11: 67-80.

Calberry, J. M., J. C. Plaizier, M. S. Einarson, and B. W. McBride. 2003. Effects of replacing chopped alfalfa hay with alfalfa silage in a total mixed ration on production and rumen conditions of lactating dairy cows. J. Dairy Sci. 86: 3611-3619.

Caprilli, R., P. Lapaquette, and A. Darfeuille-Michaud. 2010. Eating the enemy in Crohn's disease: an old theory revisited. J. Crohn's and Colitis 4: 377-383.

Carvalho, F. A., N. Barnich, P. Sauvanet, C. Darcha, A. Gelot, and A. Darfeuille-Michaud. 2008. Crohn's disease-associated *Escherichia coli* LF82 aggravates colitis in injured mouse colon via signaling by flagellin. Inflamm. Bowel. Dis. 14: 1051-1060.

Carvalho, F. A., N. Barnich, A. Sivignon, C. Darcha, C.H.F. Chan, C. P. Stanners, and A. Darfeuille-Michaud. 2009. Crohn's disease adherent-invasive *Escherichia coli* colonize and induce strong gut inflammation in transgenic mice expressing human CEACAM. J. Exp. Med. 206: 2179-2189.

Chassaing, B., N. Rolhion, A. de Vallée, S. Y. Salim, M. Prorok-Hamon,C. Neut, B. J. Campbell, J. D. Söderholm, J. P. Hugot, J. F. Colombel, and A. Darfeuille-Michaud. 2011. Crohn disease–associated adherent-invasive *E. coli* bacteria target mouse and human Peyer’s patches via long polar fimbriae. J. Clin. Invest. 121: 966-975.

Chen, X., S. Schauder, N. Potier, A. V. Dorsselaer, I. Pelczer, B. L. Bassler, and F. M. Hughson. 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415: 545-549.

Chilcott, G. S., and K. T. Hughes. 2000. Coupling of flagellar gene expression to flagellar assembly in Salmonella enterica serovar typhimurium and *Escherichia coli.* Microbiol. Mol. Biol. Rev. 64: 694-708.

Claret, L., S. Miquel, N. Vieille, D. A. Ryjenkov, M. Gomelsky, and A. Darfeuille-Michaud. 2007. The flagellar sigma factor flia regulates adhesion and invasion of Crohn disease-associated *Escherichia coli* via a cyclic dimeric GMP-dependent pathway. J. Biol. Chem. 282: 33275-33283.

Clarke, E. J., and C. A. Voigt. 2011. Characterization of combinatorial patterns generated by multiple two-component sensors in *E. coli* that respond to many stimuli. Biotechnol. Bioeng. 108: 666-675.

Clarke, M. B., D. T. Hughes, C. Zhu, E. C. Boedeker, and V. Sperandio. 2006. The *QseC* sensor kinase: a bacterial adrenergic receptor. Proc. Natl. Acad. Sci. U.S.A. 103: 10420-10425.

Clarke M.B., and V. Sperandio. 2006. LuxS dependent regulation of Escherichia coli virulence. Pages 151-174 in: Bacterial cell to cell communication role in virulence and pathogenesis. Demuth D.R and Lamont R.J. ed. Cambridge University Press, U.K.

Clermont, O., S. Bonacorsi, and E. Bingen. 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl. Environ. Microbiol. 66: 4555-4558.

Collins, S. M., and P. Bercik. 2009. The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. Gastroenterology 136: 2003-2014.

Cookson, A., W. A. Cooleyb, and M. J. Woodwarda. 2002. The role of type 1 and curli fimbriae of Shiga toxin-producing in adherence to abiotic surfaces. Intern. J. Med. Microbiol. 292: 195-205.

Costerton, J. W., K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie. 1987. Bacterial biofilms in nature and disease. Ann. Rev. Microbiol. 41: 435-464.

Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: a common cause of persistent infections. Science 284: 1318-1322.

Cui, X., and R. Harling. 2005. N-acyl-homoserine lactone-mediated quorum sensing blockage, a novel strategy for attenuating pathogenicity of Gram-negative bacterial plant pathogens. European J. Plant Pathol. 111: 327-339.

Da Re, S., B. Le Quere, J. M. Ghigo, and C. Beloin. 2007. Tight modulation of *Escherichia coli* bacterial biofilm formation through controlled expression of adhesion factors. Appl. Environ. Microbiol. 73: 3391-3403.

Darfeuille-Michaud, A. 2002. Adherent-invasive *Escherichia coli*: a putative new *E. coli* pathotype associated with Crohn's disease. Intern. J. Med. Microbiol. 292: 185-193.

Darfeuille-Michaud, A., J. Boudeau, P. Bulois, C. Neut, A. L. Glasser, N. Barnich, M. A. Bringer, A. Swidsinski, L. Beaugerie, and J. F. Colombel. 2004. High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease. Gastroenterology 127: 412-421.

Danese, P. N., L. A. Pratt, and R. Kolter. 2000. Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. J. Bacteriol. 182: 3593-3596.

Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U.S.A. 97: 6640-6645.

Davey, M. E., and G. A. O'toole. 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64: 847-867.

De Hertogh, G., J. Aerssens, K. P. Geboes, and K. Geboes. 2008. Evidence for the involvement of infectious agents in the pathogenesis of Crohn's disease. World J. Gastroenterol. 14: 845.

DeLisa, M. P., J. J. Valdes, and W. E. Bentley. 2001a. Mapping stress-induced changes in autoinducer ai-2 production in chemostat-cultivated *Escherichia coli* K-12. J. Bacteriol. 183: 2918-2928.

DeLisa, M. P., C. F. Wu, L. Wang, J. J. Valdes, and W. E. Bentley. 2001b. DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli*. J. Bacteriol. 183: 5239-5247.

Dettmer, A. M., M. A. Novak, S. J. Suomi, and J. S. Meyer. 2011. Physiological and behavioral adaptation to relocation stress in differentially reared rhesus monkeys: hair cortisol as a biomarker for anxiety-related responses. Psychoneuroendocrinology In Press, Corrected Proof.

Dewanti, R., and A. C. L. Wong. 1995. Influence of culture conditions on biofilm formation by *Escherichia coli* O157:H7. Intern. J. Food Microbiol. 26: 147-164.

Dobbin, H. S., C. J. Hovde, C. J. Williams, and S. A. Minnich. 2006. The *Escherichia coli* O157 flagellar regulatory gene flhC and not the flagellin gene fliC impacts colonization of cattle. Infect. Immun. 74: 2894-2905.

Dobrindt, U., G. Blum-Oehler, T. Hartsch, G. Gottschalk, E. Z. Ron, R. Funfstuck, and J. Hacker. 2001. S-fimbria-encoding determinant sfai is located on pathogenicity island of uropathogenic *Escherichia coli* strain 536. Infect. Immun. 69: 4248-4256.

Domka, J., J. Lee, and T. K. Wood. 2006. YliH (BssR) and YceP (BssS) regulate *Escherichia coli* K-12 biofilm formation by influencing cell signaling. Appl. Environ. Microbiol. 72: 2449-2459.

Duan K., and M. Surette. 2006. LuxS in cellular metabolism and cell to cell signaling. Pages 117-149 in: Bacterial cell to cell communication role in virulence and pathogenesis. Demuth D.R and Lamont R.J. ed. Cambridge University Press, U.K.

Epstein, W. 2003. The roles and regulation of potassium in bacteria. Prog. Nuc. Acid Res. Mol. Biol. 75: 293-320.

Erickson, D.L., V.L. Nsereko, D.P. Morgavi, L.B. Selinger, L.M. Rode, and K.A.Beauchemin. 2002. Evidence of quorum sensing in the rumen ecosystem:

detection of N-acyl homoserine lactone autoinducers in ruminal contents. Can. J. Microbiol. 48: 374-378.

Ferrieres, L., V. Hancock, and P. Klemm. 2007. Biofilm exclusion of uropathogenic bacteria by selected asymptomatic bacteriuria *Escherichia coli* strains. Microbiology 153: 1711-1719.

Fischbach, M. A., H. Lin, L. Zhou, Y. Yu, R. J. Abergel, D. R. Liu, K. N. Raymond, B. L.Wanner, R. K. Strong, C. T. Walsh, A. Aderem, and K. D. Smith. 2006. The pathogen-associated iroA gene cluster mediates bacterial evasion of lipocalin 2. Proc. Natl. Acad. Sci. U.S.A.103: 16502–16507

Fuqua, C., M. R. Parsek, and E. P. Greenberg. 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Ann. Rev. Gen. 35: 439-468.

Galperin, M. Y. 2009. Sensory transduction network of *E. coli* chapter 8. In: Systems Biology and Biotechnology of *Escherichia coli.* S.Y. Lee (ed.) Springer. Pages 134-146.

Ghia, J.-E., P. Blennerhassett, and S. M. Collins. 2008. Impaired parasympathetic function increases susceptibility to inflammatory bowel disease in a mouse model of depression. J. Clin. Invest. 118:2209–2218

Giraud, A., S. Arous, M. De Paepe, V. Gaboriau-Routhiau,J. C. Bambou, S. Rakotobe, A. B. Lindner, F. Taddei, and N. Cerf-Bensussan. 2008. Dissecting the genetic components of adaptation of *Escherichia coli* to the mouse gut. PLoS Genet 4: e2.

Glasser, A. L., J. Boudeau, N. Barnich, M. H. Perruchot, J. F. Colombel, and A. Darfeuille-Michaud1. 2001. Adherent invasive *Escherichia coli* strains from patients with Crohn's disease survive and replicate within macrophages without inducing host cell death. Infect. Immun. 69: 5529-5537.

Glasser, A. L., and A. Darfeuille-Michaud. 2008. Abnormalities in the handling of intracellular bacteria in Crohn’s disease: a link between infectious etiology and host genetic susceptibility. Arch. Immun. Therapiae Experimen. 56: 237-244.

Goller, C.C., and T. Romeo. 2008. Environmental influences on biofilm development. Pages 37-66 in: Bacterial Biofilms Current Topics in Microbiology and Immunology. T. Romeo.ed. Springer-Verlag Berlin Heidelberg.

Gonzalez Barrios, A. F., R. Zuo, Y. Hashimoto, L. Yang, W. E. Bentley, and T. K. Wood. 2006. Autoinducer 2 controls biofilm formation in *Escherichia coli*

through a novel motility quorum-sensing regulator (MqsR, B3022). J. Bacteriol. 188: 305-316.

Graff, L. A., J. R. Walker, I. Clara, L. Lix, N. Miller, L. Rogala, P. Rawsthorne, and C. N. Bernstein. 2009. Stress coping, distress, and health perceptions in inflammatory bowel disease and community controls. Am. J. Gastroenterol. 104: 2959-2969.

Gualdi, L., L. Tagliabue, S. Bertagnoli, T. Ieranò, C. De Castro, and P. Landini. 2008. Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in *Escherichia coli*. Microbiology 154: 2017-2024.

Hall, T.A. 1999. Bioedit: a user-friendly biological sequence aligment editor and analysis program for Windows 95/98/NT. Nucleic. Acid Symp. Ser. 41:95-98.

Hancock, V., and P. Klemm. 2006. Global gene expression profiling of asymptomatic bacteriuria *Escherichia coli* during biofilm growth in human urine. Infect. Immun. 75: 966-976.

Hancock, V., L. Ferrieres, and P. Klemm. 2008. The ferric yersiniabactin uptake receptor FyuA is required for efficient biofilm formation by urinary tract infectious *Escherichia coli* in human urine. Microbiology 154: 167-175.

Hannan, S. , D. Ready, A. S. Jasni, M. Rogers, J. Pratten, and A. P. Roberts. 2010. Transfer of antibiotic resistance by transformation with eDNA within oral biofilms. FEMS Immun. Med. Microbiol. 59 : 345–349.

Hardie, K. R., and K. Heurlier. 2008. Establishing bacterial communities by 'word of mouth': LuxS and autoinducer 2 in biofilm development. Nat. Rev. Microbiol. 6: 635-643.

Harshey, R. M. 2003. Bacterial motility on a surface: many ways to a common goal. Ann. Rev. Microbiol 57: 249-273.

Hassett, D. J., T. R. Korfhagen, R. T. Irvin, M. J. Schurr, K. Sauer, G. W. Lau, M. D. Sutton, H. Yu, and N. Hoiby. 2010. *Pseudomonas aeruginosa* biofilm infections in cystic fibrosis: insights into pathogenic processes and treatment strategies. Exp. Opin. Therap. Targ. 14: 117-130.

Herzberg, M., I. K. Kaye, W. Peti, and T. K. Wood. 2005. YdgG (TqsA) controls biofilm formation in *Escherichia coli* K-12 through autoinducer 2 transport. J. Bacteriol. 188: 587-598.

Holden, N. J., M. Totsika, E. Mahler, A. J. Roe, K. Catherwood, K. Lindner, U. Dobrindt, and D. L. Gally. 2006. Demonstration of regulatory cross-talk between P fimbriae

and type 1 fimbriae in uropathogenic *Escherichia coli*. Microbiology 152: 1143-1153.

Holden, N. J., and D. L. Gally. 2004. Switches, cross-talk and memory in *Escherichia coli* adherence. J. Med. Microbiol 53: 585-593.

House, B., J. V. Kus, N. Prayitno, R. Mair, L. Que, F. Chingcuanco,V. Gannon, D. G. Cvitkovitch, and D. Barnett Foster et al. 2009. Acid-stress-induced changes in enterohaemorrhagic *Escherichia coli* O157 : H7 virulence. Microbiology 155: 2907-2918.

Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2008. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Prot. 4: 44-57.

Hughes, D. T., and V. Sperandio. 2008. Inter-kingdom signalling: communication between bacteria and their hosts. Nat. Rev. Microbiol. 6: 111-120.

Hughes, D. T., M. B. Clarke, K. Yamamoto, D. A. Rasko, and V. Sperandio.2009. The *QseC* adrenergic signaling cascade in enterohemorrhagic *E. coli* (EHEC). PLoS Pathogens 5: e1000553.

Irizarry, R. A., B. M. Bolstad, F. Collin, L. M. Cope, B. Hobbs, and T. P. Speed. 2003a. Summaries of Affymetrix genechip probe level data. Nuc. Acids Res. 31: e15.

Irizarry, R. A., B. Hobbs, F. Collin, Y. D. Beazer-Barclay, K. J. Antonellis, U. Scherf, and T. P. Speed. 2003b. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4: 249-264.

Itoh, Y., J. D. Rice, C. Goller, A. Pannuri, J. Taylor, J. Meisner, T. J. Beveridge, J. F. Preston III, and T. Romeo. 2008. Roles of pgaABCD genes in synthesis, modification, and export of the *Escherichia coli* biofilm adhesin poly-{beta}-1,6-N-acetyl-D-glucosamine. J. Bacteriol. 190: 3670-3680.

Jacobsen, S. M., D. J. Stickler, H. L. T. Mobley, and M. E. Shirtliff. 2008. Complicated catheter-associated urinary tract infections due to *Escherichia coli* and proteus mirabilis. Clin. Microbiol. Rev. 21: 26-59.

Jonas, K., A. N. Edwards, R. Simm, T. Romeo, U. Römling, and Ö. Melefors. 2008. The RNA binding protein CsrA controls cyclic di-GMP metabolism by directly regulating the expression of GGDEF proteins. Mol. Microbiol. 70: 236-257.

Kaper, J. B., and V. Sperandio. 2005. Bacterial cell-to-cell signaling in the gastrointestinal tract. Infect. Immun. 73: 3197-3209.

Kendall, M. M., D. A. Rasko, and V. Sperandio. 2007. Global Effects of the Cell-to-Cell Signaling molecules autoinducer-2, autoinducer-3, and epinephrine in a luxs mutant of enterohemorrhagic *Escherichia coli*. Infect. Immun. 75: 4875-4884.

Kendall, M.M., and V. Sperandio. 2007. Quorum sensing by enteric pathogens. Curr. Opin. Gastroenterol. 23:10-5.

Khan, A. S., I. MuÈ hldorfer, V. Demuth, U. Wallner, T. K. Korhonen, and J. Hacker. 2000. Functional analysis of the minor subunits of S fimbrial adhesin (SfaI) in pathogenic *Escherichia coli*. Mol. Gen. Genetics MGG 263: 96-105.

Knight, S. D., and J. Bouckaert. 2009. Structure, function, and assembly of type 1 fimbriae. Top. Curr. Chem. 288: 67-107.

Kolenbrander, P. E., R. J. Palmer, S. Periasamy, and N. S. Jakubovics. 2010. Oral multispecies biofilm development and the key role of cell–cell distance. Nat. Rev. Microbiol. 8: 471-480.

Kostakioti, M., M. Hadjifrangiskou, J. S. Pinkner, and S. J. Hultgren. 2009. *QseC*-mediated dephosphorylation of QseB is required for expression of genes associated with virulence in uropathogenic *Escherichia coli*. Mol. Microbiol. 73: 1020-1031.

Kotlowski, R., C. N. Bernstein, S. Sepehri, and D. O. Krause. 2007. High prevalence of *Escherichia coli* belonging to the B2+D phylogenetic group in inflammatory bowel disease. Gut 56: 669-675.

Krause, D. O., A. C. Little, S. E. Dowd, and C. N. Bernstein. 2011. Complete genome sequence of adherent invasive *Escherichia coli* um146 isolated from ileal Crohn's disease biopsy tissue. J. Bacteriol. 193: 583-.

Kroes, I., P. W. Lepp, and D. A. Relman. 1999. Bacterial diversity within the human subgingival crevice. Proc. Natl. Acad. Sci. U.S.A. 96: 14547-14552.

Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17: 1244-1245.

Lacqua, A., O. Wanner, T. Colangelo, M. G. Martinotti, and P. Landini. 2006. Emergence of biofilm-forming subpopulations upon exposure of *Escherichia coli* to environmental bacteriophages. Appl. Environ. Microbiol. 72: 956-959.

Lane, M. C., V. Lockatell, G. Monterosso, D. Lamphier, J. Weinert, J. R. Hebel, D. E. Johnson, and H. L. T. Mobley. 2005. Role of motility in the colonization of uropathogenic *Escherichia coli* in the urinary tract. Infect. Immun. 73: 7644-7656.

Lane, M. C., C. J. Alteri, S. N. Smith, and H. L. T. Mobley. 2007a. Expression of flagella is coincident with uropathogenic *Escherichia coli* ascension to the upper urinary tract. Proc. Natl. Acad. Sci. U.S.A. 104: 16669-16674.

Lane, M. C., A. N. Simms, and H. L. T. Mobley. 2007b. Complex interplay between type 1 fimbrial expression and flagellum-mediated motility of uropathogenic *Escherichia coli*. J. Bacteriol. 189: 5523-5533.

Larkin, M. A., G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson, and D.G. Higgins. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948.

Laub, M. T., and M. Goulian. 2007. Specificity in two-component signal transduction pathways. Ann. Rev. Gen. 41: 121-145.

Le Quéré, B., and J.-M. Ghigo. 2009. BcsQ is an essential component of the *Escherichia coli* cellulose biosynthesis apparatus that localizes at the bacterial cell pole. Mol. Microbiol. 72: 724-740.

Lim, J. Y., H. J. La, H. Sheng, L. J. Forney, and C. J. Hovde. 2009. Influence of plasmid pO157 on *Escherichia coli* O157:H7 sakai biofilm formation. Appl. Environ. Microbiol.76: 963-966.

Mahajan, A., C. G. Currie, S. Mackie, J. Tree, S. McAteer, I. McKendrick, T. N. McNeilly, A. Roe, R. M. La Ragione, M. J. Woodward, D. L. Gally, and D. G. E. Smith. 2009. An investigation of the expression and adhesin function of H7 flagella in the interaction of *Escherichia coli* O157:H7 with bovine intestinal epithelium. Cell. Microbiol. 11: 121-137.

Martin, H. M., B. J. Campbell, C. A. Hart, C. Mpofu, M. Nayar, R. Singh, H. Englyst, H. F. Williams, and J. M. Rhodes. 2004. Enhanced *Escherichia coli* adherence and invasion in Crohn's disease and colon cancer. Gastroenterology 127: 80-93.

Martinez-Medina, M., A. Mora, M. Blanco, C. Lo´pez, M. P. Alonso, S. Bonacorsi, M. H. Nicolas-Chanoine, A. Darfeuille-Michaud, J. Garcia-Gil, and J. Blanco. 2009a. Similarity and divergence among adherent-invasive *Escherichia coli* and extraintestinal pathogenic *E. coli* strains. J. Clin. Microbiol. 47: 3968-3979.

Martinez-Medina, M., P. Naves, J. Blanco, X. Aldeguer, J. E. Blanco, M. Blanco, C. Ponte, F. Soriano, A. Darfeuille-Michaud, and L. J. Garcia-Gil. 2009b. Biofilm formation as a novel phenotypic feature of adherent-invasive *Escherichia coli* (AIEC). BMC Microbiol. 9: 202.

Melican, K., R. M. Sandoval, A. Kader, L. Josefsson, G. A. Tanner, B. A. Molitoris, A. Richter-Dahlfors. 2011. Uropathogenic *Escherichia coli* P and type 1 fimbriae act

in synergy in a living host to facilitate renal colonization leading to nephron obstruction. PLoS Pathog 7: e1001298.

Methner, U., W. Rabsch, R. Reissbrodt, and P. Williams. 2008. Effect of norepinephrine on colonisation and systemic spread of *Salmonella enterica* in infected animals: Role of catecholate siderophore precursors and degradation products. Intern. J. Med. Microbiol. 298: 429-439.

Miller, M. B., and B. L. Bassler. 2001. Quorum sensing in bacteria. Ann. Rev. Microbiol. 55: 165-199.

Mitsumori, M., L. Xu, H. Kajikawa, M. Kurihara, K. Tajima, J. Hai, A. Takenaka. 2003. Possible quorum sensing in the rumen microbial community: detection of quorum-sensing signal molecules from rumen bacteria. FEMS Microbiol. Lett. 219: 47-52.

Monday, S. R., S. A. Minnich, and P. C. H. Feng. 2004. A 12-Base-pair deletion in the flagellar master control gene flhC causes nonmotility of the pathogenic german sorbitol-fermenting *Escherichia coli* O157:H- strains. J. Bacteriol. 186: 2319-2327.

Monteiro, C., I. Saxena, X. Wang, A. Kader, W. Bokranz, R. Simm, D. Nobles, M. Chromek, A. Brauner, R. M. Brown Jr, and U. Römling. 2009. Characterization

of cellulose production in *Escherichia coli* Nissle 1917 and its biological consequences. Environ. Microbiol. 11: 1105-1116.

Moore, F. R., E.A.S. Al Dujaili, R.E. Cornwell, M.J. Law Smith, J.F. Lawson, M. Sharp, D.I. Perrett. 2011. Cues to sex- and stress-hormones in the human male face: Functions of glucocorticoids in the immunocompetence handicap hypothesis. Hormones and Behavior In Press, Corrected Proof.

Mulvey, M. A. 2002. Adhesion and entry of uropathogenic *Escherichia coli*. Cell. Microbiol. 4: 257-271.

Nataro, J. P., and J. B. Kaper. 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11: 403-.

Naves, P., G. del Prado, L. Huelves, M. Gracia, V. Ruiz, J. Blanco, G. Dahbi, M. Blanco, M. C. Ponte, and F. Soriano. 2008. Correlation between virulence factors and in vitro biofilm formation by *Escherichia coli* strains. Microbial Pathogenesis 45: 86-91.

Nishino, K., T. Honda, and A. Yamaguchi. 2005. Genome-wide analyses of *Escherichia coli* gene expression responsive to the BaeSR two-component regulatory system. J. Bacteriol. 187: 1763-1772.

Ogura, Y., D. K. Bonen, N. Inohara, D. L. Nicolae, F. F. Chen, R. Ramos, H. Britton, T. Moran, R. Karaliuskas, R. H. Duerrk, J. P. Achkar, S. R. Brant, T. M. Bayless, B. S. Kirschner, S. B. Hanauer, G. Nuñez, and J. H. Cho. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 411: 603-606.

Ong, C.-L. Y., G. C. Ulett, A. N. Mabbett, S. A. Beatson, R. I. Webb, W. Monaghan, G. R. Nimmo, D. F. Looke, A. G. McEwan, and M. A. Schembri. 2008. Identification of type 3 fimbriae in uropathogenic *Escherichia coli* reveals a role in biofilm formation. J. Bacteriol. 190: 1054-1063.

Orholm, M., P. Munkholm, E. Langholz, O. H. Nielsen, T. I. A. Sørensen, and V. Binder. 1991. Familial occurrence of inflammatory bowel disease. New England J. Med. 324: 84-88.

Orholm, M., T. I. A. Sørensen, L. P. Rasmussen, K. O. Kyvik. 2000. Concordance of inflammatory bowel disease among danish twins: results of a nationwide study. Scan. J. Gastroenterol. 35: 1075-1081.

Orndorff, P. E., A. Devapali, S. Palestrant, A. Wyse, M. L. Everett, R. R. Bollinger, and W. Parker. 2004. Immunoglobulin-mediated agglutination of and biofilm formation by *Escherichia coli* K-12 require the type 1 pilus fiber. Infect. Immun. 72: 1929-1938.

Oshima, T., H. Aiba,Y. Masuda, S. Kanaya, M. Sugiura, B. L. Wanner, H. Mori, and T. Mizuno. 2002. Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. Mol. Microbiol. 46: 281-291.

Pacheco, A. R., and V. Sperandio. 2009. Inter-kingdom signaling: chemical language between bacteria and host. Curr. Opin. Microbiol. 12: 192-198.

Parker, C. T., and V. Sperandio. 2009. Cell-to-cell signalling during pathogenesis. Cell. Microbiol. 11: 363-369.

Pichon, C., C. Héchard, L. Merle, C. Chaudray, I. Bonne, S. Guadagnini, A. Vandewalle, and C. L. Bouguénec1. 2009. Uropathogenic *Escherichia coli* AL511 requires flagellum to enter renal collecting duct cells. Cell. Microbiol. 11: 616-628.

Pihlstrom, B. L., B. S. Michalowicz, and N. W. Johnson. 2005. Periodontal diseases. The Lancet 366: 1809-1820.

Poritsanos, N.J. 2006. Molecular mechanisms involved in secondary metabolite production and biocontrol of *Pseudomonas chlororaphis* PA23. M.Sc. Thesis. The University of Manitoba, Winnipeg, Canada.

Pratt, L. A., and R. Kolter. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. 30: 285-293.

Pratt, L. A., and T. J. Silhavy. 1995. Porin regulon of *Escherichia coli*. Pages 105-127 in: Two Component Signal Transduction. J. A. Hoch, and T. J. Silhavy. ed. ASM press. Washington, D.C.

Prigent-Combaret, C., E. Brombacher, O. Vidal, A. Ambert, P. Lejeune, P. Landini, and C. Dorel. 2001. Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the csgD gene. J. Bacteriol. 183: 7213-7223.

Pruss, B. M., C. Besemann, A. Denton, and A. J. Wolfe. 2006. A complex transcription network controls the early stages of biofilm development by *Escherichia coli*. J. Bacteriol. 188: 3731-3739.

Ravi, M., M. Ngeleka, S. H. Kim, C. Gyles, F. Berthiaume, M. Mourez, D. Middleton, E. Simko. 2007. Contribution of AIDA-I to the pathogenicity of a porcine diarrheagenic *Escherichia coli* and to intestinal colonization through biofilm formation in pigs. Vet. Microbiol. 120: 308-319.

Raymond, K. N., E. A. Dertz, and S. S. Kim 2003. Bioinorganic chemistry special feature: enterobactin: an archetype for microbial iron transport. Proc. Natl. Acad. Sci. U.S.A. 100: 3584-3588.

Reading, N. C., D. A. Rasko, A. G. Torres, and V. Sperandio. 2009. The two-component system QseEF and the membrane protein QseG link adrenergic and stress sensing to bacterial pathogenesis. Proc. Natl. Acad. Sci. U.S.A. 106: 5889-5894.

Reinstein, S., J. T. Fox, X. Shi, M. J. Alam, D. G. Renter, and T. G. Nagaraja. 2009. Prevalence of *Escherichia coli* O157:H7 in organically and naturally raised beef cattle. Appl. Environ. Microbiol.75: 5421-5423.

Reisner, A., K. A. Krogfelt, B. M. Klein, E. L. Zechner, and S. Molin. 2006b. In vitro biofilm formation of commensal and pathogenic *Escherichia coli* strains: impact of environmental and genetic factors. J. Bacteriol. 188: 3572-3581.

Ren, D., L. A. Bedzyk, S. M. Thomas, R. W. Ye, and T. K. Wood. 2004. Gene expression in *Escherichia coli* biofilms. Appl. Microbiol. Biotechnol. 64: 515-524.

Rezzonico, F., and B. Duffy. 2008. Lack of genomic evidence of AI-2 receptors suggests a non-quorum sensing role for *luxS* in most bacteria. BMC Microbiol. 8:154.

Rolhion, N., N. Barnich, M. A. Bringer, A. L. Glasser, J. Ranc, X. He´buterne, P. Hofman, and A. Darfeuille-Michaud. 2010. Abnormally expressed ER stress response chaperone Gp96 in CD favours adherent-invasive *Escherichia coli* invasion. Gut 59: 1355-1362.

Rolhion, N., F. A. Carvalho, and A. Darfeuille-Michaud. 2007. OmpC and the σE regulatory pathway are involved in adhesion and invasion of the Crohn's disease-associated *Escherichia coli* strain LF82. Mol. Microbiol. 63: 1684-1700.

Rolhion, N., and A. Darfeuille-Michaud. 2007. Adherent-invasive *Escherichia coli* in inflammatory bowel disease. Inflamm. Bowel. Dis. 13: 1277-1283.

Römling, U. 2002. Molecular biology of cellulose production in bacteria. Research in Microbiology 153: 205-212.

Römling, U. 2005. Characterization of the rdar morphotype, a multicellular behaviour in Enterobacteriaceae. Cell. Mol. Life Sci. 62: 1234-1246.

Rowe, M. C., H. L. Withers, and S. Swift. 2010. Uropathogenic *Escherichia coli* forms biofilm aggregates under iron restriction that disperse upon the supply of iron. FEMS Microbiol. Lett. 307: 102-109.

Ryu, J. H., and L. R. Beuchat. 2005. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: effect of exopolysaccharide and curli production on its resistance to chlorine. Appl. Environ. Microbiol. 71: 247-254.

Saldaña, Z., J. Xicohtencatl-Cortes, F. Avelino, A. D. Phillips, J. B.Kaper, J. L. Puente, and J. A. Girón. 2009. Synergistic role of curli and cellulose in cell adherence and biofilm formation of attaching and effacing *Escherichia coli* and identification of Fis as a negative regulator of curli. Environ. Microbiol. 11: 992-1006.

Sambrook, L., and D.W. Russell. 2001. Molecular cloning: a laboratory manual 3rd Ed. Cold Spring Laboratory Press, Cold Spring Harbor, USA.

Sanchez-Torres, V., H. Hu, and T. K. Wood. 2010. GGDEF proteins YeaI, YedQ, and YfiN reduce early biofilm formation and swimming motility in *Escherichia coli*. Appl. Microbiol. Biotechnol. 90: 651-658.

Sartor, R. B. 2008. Microbial Influences in inflammatory bowel disease. Gastroenterology 134: 577-594.

SAS Institute., 1996. SAS system for mixed models. 4th ed. SAS Inst., Inc., Cary NC.

Schauder, S., K. Shokat, M. G. Surette, and B. L. Bassler. 2001. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol. Microbiol. 41: 463-476.

Schembri, M. A., K. Kjærgaard, and P. Klemm. 2003. Global gene expression in *Escherichia coli* biofilms. Mol. Microbiol. 48: 253-267.

Schembri, M. A., and P. Klemm. 2001a. Biofilm formation in a hydrodynamic environment by novel fimh variants and ramifications for virulence. Infect. Immun. 69: 1322-1328.

Schembri, M. A., and P. Klemm. 2001b. Coordinate gene regulation by fimbriae-induced signal transduction. EMBO J. 20: 3074-3081.

Schmoll, T. , H. Hoschiitzky, J. Morschhauser, F. Lottspeich, K. Jann, and J. Hacker. 1989. Analysis of genes coding for the sialic acid-binding adhesin and two other minor fimbrial subunits of the S-fimbrial adhesin determinant of *Escherichia coli*. Mol. Microbiol. 3: 1735-1744.

Schwan, W. R. 2009. Survival of uropathogenic *Escherichia coli* in the murine urinary tract is dependent on OmpR. Microbiology 155: 1832-1839.

Sepehri, S., R. Kotlowski, C. N. Bernstein, and D. O. Krause. 2009. Phylogenetic analysis of inflammatory bowel disease associated *Escherichia coli* and the FimH virulence determinant. Inflamm. Bowel. Dis.s 15: 1737-1745.

Setia, A. 2007. Selection of *Escherichia coli* K88+ specific probiotic strains of *E. coli* from environmental isolates for post-weaning piglets. M.Sc. Thesis, The University of Manitoba, Winnipeg, Canada.

Sharp, F. C., and V. Sperandio. 2007. QseA directly activates transcription of LEE1 in enterohemorrhagic *Escherichia coli*. Infect. Immun. 75: 2432-2440.

Sheng, H., J. Y. Lim, H. J. Knecht, J. Li, and C. J. Hovde. 2006. Role of *Escherichia coli* O157:H7 virulence factors in colonization at the bovine terminal rectal mucosa. Infect. Immun. 74: 4685-4693.

Sherlock, O., M. A. Schembri, A. Reisner, and P. Klemm. 2004. Novel roles for the aida adhesin from diarrheagenic *Escherichia coli*: cell aggregation and biofilm formation. J. Bacteriol. 186: 8058-8065.

Shin, S., and C. Park. 1995. Modulation of flagellar expression in *Escherichia coli* by acetyl phosphate and the osmoregulator OmpR. J. Bacteriol. 177: 4696-4702.

Simms, A. N., and H. L. T. Mobley. 2008a. multiple genes repress motility in uropathogenic *Escherichia coli* constitutively expressing type 1 fimbriae. J. Bacteriol. 190: 3747-3756.

Simms, A. N., and H. L. T. Mobley. 2008b. PapX, a P fimbrial operon-encoded inhibitor of motility in uropathogenic *Escherichia coli*. Infect. Immun. 76: 4833-4841.

Singh, S. A. Blanchard, J. R. Walker, L. A. Graff, N. Miller, and C. N. Bernstein. 2011. Common symptoms and stressors among individuals with inflammatory bowel diseases. Clin. Gastroenterol. Hepatol. In Press, Uncorrected Proof.

Sjöström, A. E., C. Balsalobre, L. Emo¨dy, B. Westerlund-Wikstro¨mc, J. Hacker, B. E. Uhlin. 2009. The SfaXII protein from newborn meningitis *E. coli* is involved in regulation of motility and type 1 fimbriae expression. Microb. Pathogen. 46: 243-252.

Skandamis, P. N., J. D. Stopforth, L. V. Ashton, I. Geornaras, P.A. Kendall, J. N. Sofos. 2009. *Escherichia coli* O157:H7 survival, biofilm formation and acid tolerance under simulated slaughter plant moist and dry conditions. F. Microbiol. 26: 112-119.

Skyberg, J. A., K. E. Siek, C. Doetkott, and L. K. Nolan. 2007. Biofilm formation by avian *Escherichia coli* in relation to media, source and phylogeny. J. Appl. Microbiol. 102 :548–554.

Soutourina, O. A., and P. N. Bertin. 2003. Regulation cascade of flagellar expression in Gram-negative bacteria. FEMS Microbiol. Rev. 27: 505-523.

Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper. 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 96: 15196-15201.

Sperandio, V., A. G. Torres, B. Jarvis, J. P. Nataro, and J. B. Kaper 2003. Bacteria-host communication: the language of hormones. Proc. Natl. Acad. Sci. U.S.A. 100: 8951-8956.

Sperandio, V., A. G. Torres, J. A. Giron, and J. B. Kaper. 2001. Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. J. Bacteriol. 183: 5187-5197.

Sperandio, V., A. G. Torres, and J. B. Kaper. 2002. Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved

in the regulation of flagella and motility by quorum sensing in *E. coli*. Mol. Microbiol. 43: 809-821.

Stepanović, S., I. Ćirković, L. Ranin, and M. S. Svabic´-Vlahovic. 2004. Biofilm formation by *Salmonella spp*. and *Listeria monocytogenes* on plastic surface. Lett. Appl. Microbiol. 38: 428-432.

Sun, L., G. M. Nava, and T. S. Stappenbeck. 2011. Host genetic susceptibility, dysbiosis, and viral triggers in inflammatory bowel disease. Curr. Opin. Gastroenterol. 27: 321-327.

Surette, M. G., and B. L. Bassler. 1998. Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. Proc. Natl. Acad. Sci. U.S.A. 95: 7046-7050.

Surette, M. G., M. B. Miller, and B. L. Bassler. 1999. Quorum sensing in *Escherichia coli*, *Salmonella typhimurium,* and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci. U.S.A. 96: 1639-1644.

Taché, Y., C. Kiank, and A. Stengel. 2009. A role for corticotropin-releasing factor in functional gastrointestinal disorders. Curr. Gastroenterol. Rep. 11: 270-277.

Tatusova, T. A., and T. L. Madden. 1999. BLAST 2 S, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol. Lett. 174: 247-250.

Ter Braak, C.J.F., and P. Smilauer. 2002. CANOCO reference manual and CanocoDraw for windows user’s guide: software for canonical community ordination. Version 4.5. Microcomputer power Ithaca, NY, USA.

Torres, A. G., and S. M. Payne. 1997. Haem iron-transport system in enterohaemorrhagic Escherichia coli O157:H7. Mol. Microbiol. 23: 825-833.

Tysk, C., E. Lindberg, G. Järnerot, and B. Flodérus-Myrhed. 1988. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. a study of heritability and the influence of smoking. Gut 29: 990-996.

Uhlich, G. A., P. H. Cooke, and E. B. Solomon. 2006. Analyses of the red-dry-rough phenotype of an *Escherichia coli* O157:H7 strain and its role in biofilm formation and resistance to antibacterial agents. Appl. Environ. Microbiol. 72: 2564-2572.

Ulett, G. C., A. N. Mabbett, K. C. Fung, R. I. Webb, and M. A. Schembri. 2007a. The role of F9 fimbriae of uropathogenic *Escherichia coli* in biofilm formation. Microbiology 153: 2321-2331.

Ulett, G. C., J. Valle, C. Beloin, O. Sherlock, J. M. Ghigo, and M. A. Schembri. 2007b. Functional analysis of antigen 43 in uropathogenic *Escherichia coli* reveals a role in long-term persistence in the urinary tract. Infect. Immun. 75: 3233-3244.

Valle, J., S. Da Re, S. Schmid, D. Skurnik, R. D'Ari, and J. M. Ghigo. 2007. The amino acid valine is secreted in continuous-flow bacterial biofilms. J. Bacteriol. 190: 264-274.

Vanhoudt, R., and C. Michiels. 2005. Role of bacterial cell surface structures in biofilm formation. Res. Microbiol. 156: 626-633.

Virkola, R., B. Westerlund, H. Holthofer, J. Parkkinen, M. Kekomaki, and T. K. Korhonen. 1988. Binding characteristics of *Escherichia coli* adhesins in human urinary bladder. Infect. Immun. 56: 2615-2622.

Visick, K. L., and M. J. McFall-Ngai. 2000. An exclusive contract: specificity in the *Vibrio fischeri-Euprymna scolopes* partnership. J. Bacteriol. 182: 1779-1787.

Wagner V.E., and B.H. Iglewski. 2006. Quorum sensing and regulation of *Pseudomonas aeruginosa* infections. Pages 1-22 in Bacterial cell to cell communication role in virulence and pathogenesis. Demuth D.R and Lamont R.J. ed. Cambridge University Press, U.K.

Walkty, J. 2007. Potential for pathogen transfer from hog manure fertilizer to grazed cattle and ground water. M.Sc. Thesis, The University of Manitoba, Winnipeg, Canada.

Walters, M., M. P. Sircili, and V. Sperandio. 2006. AI-3 synthesis is not dependent on *luxS* in *Escherichia coli*. J. Bacteriol. 188: 5668-5681.

Walters, M., and V. Sperandio. 2006. Autoinducer 3 and epinephrine signaling in the kinetics of locus of enterocyte effacement gene expression in enterohemorrhagic *Escherichia coli*. Infect. Immun. 74: 5445-5455.

Wang, X., H. Lünsdorf, I. Ehrén, A. Brauner, and U. Römling. 2009. Characteristics of biofilms from urinary tract catheters and presence of biofilm-related components in *Escherichia coli*. Curr. Microbiol. 60: 446-453.

Waters C.M., and Bassler B.L. 2006. The Vibrio harveyi quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. Gen. Develop. 20:2754-2767.

Weber, H., C. Pesavento, A. Possling, G. Tischendorf, and R. Hengge. 2006. Cyclic-di-GMP-mediated signalling within the network of *Escherichia coli.* Mol. Microbiol. 62: 1014-1034.

White-Ziegler, C. A., S. Um, N. M. Pe´rez, A. L. Berns, A. J. Malhowski, and S. Young. 2008. Low temperature (23 ^{0}C) increases expression of biofilm-, cold-shock- and RpoS-dependent genes in *Escherichia coli* K-12. Microbiology 154: 148-166.

Wood, T. K. 2009. Insights on *Escherichia coli* biofilm formation and inhibition from whole-transcriptome profiling. Environ. Microbiol. 11: 1-15.

Wood, T.K., and W.E. Bentley. 2007. Signaling in *Escherichia coli* biofilms. Pages 123-139 in The biofilm Mode of Life Mechanism and Adaptations. Kjellebrg, S., and Givskov, M. ed. Wymondham, U.K: Horizon Bioscience.

Wood, T. K., A. F. González Barrios, M. Herzberg, and J. Lee. 2006. Motility influences biofilm architecture in *Escherichia coli*. Appl. Microbiol. Biotechnol. 72: 361-367.

Xavier, K. B., and B. L. Bassler. 2003. LuxS quorum sensing: more than just a numbers game. Curr. Opin. Microbiol. 6: 191-197.

Xavier, K. B., and B. L. Bassler. 2005. Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*. J. Bacteriol. 187: 238-248.

Yamamoto, T., K. Fujita, and T. Yokota. 1990. Adherence characteristics to human small intestinal mucosa of *Escherichia coli* isolated from patients with diarrhea or urinary tract infections. J. Infec. Dis. 162: 896-908.

Yang, H., J. B. Morrow, D. Grasso, R.T. Vinopal, and B.F. Smets. 2006. Intestinal versus external growth conditions change the surficial properties in a collection of environmental *Escherichia coli*. Environ. Sci. Technol. 40:6976-6982.

Zhou, L., X. H. Lei, B. R. Bochner, and B. L. Wanner. 2003. Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. J. Bacteriol. 185: 4956-4972.

Zoetendal, E. G., A. H. Smith, M. A. Sundset, and R. I. Mackie. 2007. The BaeSR two-component regulatory system mediates resistance to condensed tannins in *Escherichia coli.* Appl. Environ. Microbiol. 74: 535-539.

APPENDIX

Manuscript III

List of genes expressed during AIEC biofilm formation (generated by DAVID database), the biofilms were grown on plastic surfaces using a minimal medium (M9) supplemented with glucose and casamino acids and the presence of Epi at 100 µM/ml.

	symbol	Fold change(log 2) 146 vs. 146+Epi	ΔqseC vs. ΔqseC+Epi	ΔqseB vs. ΔqseB+Epi
sugar symporter activity				
Gene Name				
ABC transporter periplasmic-binding protein yphF	yphF	1.33	NS	0.43
ABC transporter periplasmic-binding protein ytfQ	ytfQ	0.71	0.4	1.05
Capsule polysaccharide export inner-membrane protein kpsE	kpsE	1.11	-0.22	0.25
Fructose-like permease IIC component	c2925	0.9	NS	0.52
Inner membrane ABC transporter permease protein yjfF	yjfF	1.19	-0.19	1.43
Multiphosphoryl transfer protein	c2922	1.11	NS	0.55
Multiphosphoryl transfer protein 2	ptsA	1.46	0.31	0.25
N,N'-diacetylchitobiose-specific phosphotransferase enzyme	celA	1.2	0.54	-0.39
PTS system N-acetylglucosamine-specific EIICBA component	nagE	0.9	0.85	0.84
PTS system N-acetylmuramic acid-specific EIIBC component	murP	1.1	-0.2	0.38
PTS system beta-glucoside-specific EIIBCA component	bglF	1.22	0.15	-0.13
PTS system mannitol-specific EIICBA component	mtlA	0.58	2.7	1.74
PTS system mannitol-specific cryptic EIICB component	cmtA	0.88	NS	-0.67
Phosphoenolpyruvate-protein phosphotransferase	ptsI	0.87	1.67	0.32
Probable ABC transporter permease protein yphD	yphD	0.9	NS	0.25
Putative ABC transporter periplasmic-binding protein ycjN	ycjN	1.22	-0.17	0.2
Putative outer membrane porin bglH	c4642	1.2	NS	0.18
Putative sialic acid transporter 2	c3637	1.48	-0.59	0.38
Putative sialic acid transporter; Putative sialic acid transporter 1	nanT	1.22	NS	0.38
UTI89_C1930; Z2767; c2136; APECO1_806	celB	1.11	0.22	0.3
c0335	c0335	1.17	0.22	0.5

c0336	c0336	1.27	-0.34	0.32
c1956	c1956	1.14	0.11	0.84
c1957	c1957	1.1	-0.45	0.72
c1958	c1958	1.22	-0.54	NS
c3408	c3408	1.31	0.33	0.38
c4277	c4277	1.45	-0.48	0.28
c4278	c4278	1.46	0.29	NS
c4486	c4486	2.98	NS	-0.2
c4487	c4487	1.45	NS	0.72
c4488	c4488	1.45	0.3	0.49
c4758	c4758	1.42	-0.46	0.89
sn-glycerol-3-phosphate import ATP-binding protein ugpC	ugpC	1.2	1.13	0.75
glutathione metabolism				
6-phosphogluconate dehydrogenase, decarboxylating	gnd	0.82	0.98	NS
Aminoacyl-histidine dipeptidase	pepD	0.88	0.35	NS
Aminopeptidase N	pepN	0.88	1	0.25
Bifunctional glutathionylspermidine synthetase/amidase	gsp	1.12	1.42	0.44
Gamma-glutamyltranspeptidase	ggt	1.12	0.48	-0.45
Glutamate--cysteine ligase	gshA	1.2	-0.23	0.16
Glutathione reductase	gor	0.73	-2.35	-1.19
Glutathione synthetase	gshB	0.78	0.19	-0.81
Ornithine decarboxylase, inducible	speF	0.96	-2.7	0.31
Peptidase B	pepB	0.59	2.28	1.24
viron assembly				
c0948	c0948	0.83	-0.45	-0.13
c0950	c0950	1.33	-0.23	-0.2
c0953	c0953	1.74	-0.2	0.19
c0954	c0954	1.23	-0.23	0.18

c1453	c1453	1.91	-0.09	0.41
c1577	c1577	1.5	0.11	0.86
nucleotide excision repair				
DNA helicase II	uvrD	1.18	-0.7	0.32
DNA ligase B	ligB	0.93	-0.12	0.22
Transcription-repair-coupling factor	mfd	0.9	1.13	0.39
UvrABC system protein A	uvrA	1.15	0.36	0.62
UvrABC system protein B	uvrB	0.88	2.08	1.26
DNA-3-methyladenine glycosylase 2	alkA	1.62	-0.35	NS
Single-stranded-DNA-specific exonuclease recJ	recJ	1.09	-0.27	0.39
Uracil-DNA glycosylase	ung	1.19	NS	-0.25
Valine, leucine and isoleucine biosynthesis				
2-isopropylmalate synthase	leuA	0.66	-1.48	-1.31
3-isopropylmalate dehydrogenase	leuB	1.13	0.73	0.55
Acetolactate synthase isozyme 3 large subunit; Acetolactate synthase	ilvI	1.18	NS	-0.95
Dihydroxy-acid dehydratase	ilvD	1.81	0.2	-0.09
Isoleucyl-tRNA synthetase	ileS	0.89	2.06	0.4
Ketol-acid reductoisomerase	ilvC	1.11	0.93	NS
Threonine dehydratase biosynthetic	ilvA	0.81	0.6	0.49
Valine--pyruvate aminotransferase	avtA	1.31	-0.79	0.16
Valyl-tRNA synthetase	valS	1.2	0.23	0.62
Valine, leucine and isoleucine degradation				
3-ketoacyl-CoA thiolase	fadA	0.95	0.34	0.16
4-aminobutyrate aminotransferase	gabT	1.68	NS	1
Fatty acid oxidation complex subunit alpha	fadJ	1.19	-0.14	0.25
Fatty acid oxidation complex subunit alpha	fadB	1.12	0.31	0.41
ABC transporter				
APECO1_274; pECS88_0022; ECIAI39_1919; pO103_10	sitA	0.48	-5.57	-2.97

Aliphatic sulfonates import ATP-binding protein ssuB	ssuB	1.96	-0.29	0.2
Dipeptide transport ATP-binding protein dppD	dppD	1.45	0.49	1.07
Dipeptide transport system permease protein dppB	dppB	0.79	-0.6	-0.39
Dipeptide transport system permease protein dppC	dppC	1.58	NS	-0.15
ECDH10B_2287; ECS88_2275; APECO1_4418; Z3380; ECIAI1_2209;	yehZ	0.37	-0.44	-1.02
ECIAI39_3445; ECUMN_3431; c3697; UTI89_C3374; ECED1_3601;	kpsT	1.33	-0.18	0.36
ECIAI39_3446; ECED1_3602; c3698; APECO1_3468	kpsM	1.37	-1.29	NS
ECUMN_4002; c4313; E2348C_3744; ECED1_4180; ECIAI39_4004; Z4913	chuT	0.87	-1.56	-1.22
EcolC_3589; ECUMN_0069; EC55989_0066; Z0077	tbpA	1.59	-0.26	0.28
Galactoside transport system permease protein mglC	mglC	0.9	-0.72	0.18
Glycine betaine/L-proline transport ATP-binding protein proV	proV	1.17	-0.49	-0.14
High-affinity branched-chain amino acid transport ATP-binding	livF	1.11	NS	-0.07
Leu/Ile/Val-binding protein	livJ	0.83	0.12	-0.07
Leucine-specific-binding protein	livK	1.07	-1.21	-1.15
Lipoprotein-releasing system transmembrane protein lolC	lolC	1.18	-2.05	-0.74
Lipoprotein-releasing system transmembrane protein lolE	lolE	1.69	NS	-0.38
Maltose-binding periplasmic protein	malE	1.45	-0.45	NS
Molybdate-binding periplasmic protein	modA	0.64	-0.18	-0.25
Molybdenum transport system permease protein modB	modB	0.86	NS	NS
Nickel import ATP-binding protein nikE	nikE	0.88	-0.09	0.45
Peptide transport periplasmic protein sapA	sapA	0.83	-0.44	NS
Phosphate transport system permease protein pstC	pstC	0.72	0.83	0.63
Phosphonates import ATP-binding protein phnC	phnC	1.52	0.19	-0.15
Phosphonates transport ATP-binding protein phnK	phnK	1.08	0.99	-0.2
Phosphonates transport ATP-binding protein phnL	phnL	0.9	-0.43	NS
Phosphonates-binding periplasmic protein	phnD	1.26	-0.88	0.18
Putative molybdenum transport ATP-binding protein modF	modF	0.86	-0.11	0.3

Putrescine transport ATP-binding protein potG	potG	1.11	0.55	0.19
Putrescine-binding periplasmic protein	potF	1.34	0.12	-0.31
Sulfate transport system permease protein cysT	cysU	0.88	-0.68	0.46
Sulfate-binding protein	sbp	0.71	-0.39	-0.6
Sulfate/thiosulfate import ATP-binding protein cysA	cysA	1.32	-0.07	-0.07
Thiamine transport system permease protein thiP	thiP	1.2	-0.44	-0.07
Uncharacterized ABC transporter ATP-binding protein yejF	yejF	1.27	-0.87	0.25
Vitamin B12 import ATP-binding protein btuD	btuC	0.69	0.23	-1.47
c5109; ECH74115_5616; ECIAI1_4333; ECUMN_4635	phnE	1.33	0.14	0.36
pECS88_0020; APECO1_272; ECED1_1294; c1598; MM1_0108	sitC	0.91	NS	1.3
pECS88_0021; APECO1_273; pO103_11; c1599; ECS88_1216	sitB	1.09	-2.11	0.32
pO103_13; APECO1_271; ECUMN_1438; ECED1_1293; c1597	sitD	0.78	0.93	0.86
sn-glycerol-3-phosphate-binding periplasmic protein ugpB	ugpB	0.69	0.66	1.8
glutamate synthase activity				
Glutamate synthase [NADPH] large chain	gltB	1.1	1.4	0.82
Glutamate synthase [NADPH] small chain	gltD	1.66	0.09	-0.17
Uncharacterized oxidoreductase yeiT	yeiT	0.55	1.92	2.15
Uncharacterized protein ygfT	ygfT	0.86	NS	0.08
Asparagine synthetase B [glutamine-hydrolyzing]	asnB	0.78	-1.14	-2.74
Glucosamine--fructose-6-phosphate aminotransferase [isomerizing]	glmS	0.89	2.72	1.25
gly-ser-thre-metabolism				
2-amino-3-ketobutyrate coenzyme A ligase	kbl	0.88	0.27	0.4
Betaine aldehyde dehydrogenase	betB	0.53	NS	0.49
Bifunctional aspartokinase/homoserine dehydrogenase 2	metL	1.14	1.11	-0.4
D-serine dehydratase; D-serine dehydratase 1	dsdA	0.48	NS	-0.6
Glycine dehydrogenase [decarboxylating]	gcvP	0.78	0.8	0.15
Phosphoserine aminotransferase	serC	0.9	0.07	-0.82
Phosphoserine phosphatase	serB	0.61	-1.12	-0.9

Tryptophan synthase beta chain	trpB	0.94	1.58	0.13
USG-1 protein	usg	0.91	1.15	0.46
mismatch repair				
DNA mismatch repair protein mutL	mutL	0.78	0.23	0.53
DNA mismatch repair protein mutS	mutS	1.2	-0.58	-0.39
DNA polymerase III subunit alpha	dnaE	0.87	-0.53	0.55
Exodeoxyribonuclease 7 large subunit	xseA	0.9	-2.41	-1.81
lysine biosynthesis				
Succinylornithine transaminase; Acetylornithine	argD	1.09	-0.72	0.28
UDP-N-acetylmuramoyl-L-alanyl-D-glutamate	murE	0.82	1.08	0.3
lysine degradation				
2-oxoglutarate dehydrogenase E1 component	sucA	1.2	0.32	NS
L-lysine 6-monooxygenase	iucD	1.38	0.53	0.15
Lysine decarboxylase, constitutive	ldcC	1.34	0.74	0.8
virion part				
P21 prophage-derived major head protein	c1575	1.18	0.62	-0.2
c0952	c0952	1.1	-0.77	-0.59
c1447	c1447	0.85	0.59	1.2
c1450	c1450	1.79	0.57	0.55
metal ion binding site				
Phosphoglucosamine mutase	glmM	0.61	0.72	0.56
Phosphomannomutase	cpsG	2.37	0.4	0.64
propanoate metabolism				
Keto-acid formate acetyltransferase	tdcE	1.27	-0.16	0.13
Methylisocitrate lyase	prpB	1.36	-0.19	-0.46
Phosphate acetyltransferase	pta	1.08	0.47	0.19
Propionate kinase	tdcD	1.33	-0.18	NS
Propionate--CoA ligase	prpE	1.25	-0.27	0.18

Putative formate acetyltransferase 3	ybiW	0.92	0.31	0.89
viral reproduction				
Long-chain fatty acid transport protein	fadL	1.54	0.29	NS
Nucleoside-specific channel-forming protein tsx	tsx	0.62	0.21	0.58
Vitamin B12 transporter btuB	btuB	0.79	-0.11	0.45
cell wall macromolecule biosynthetic process				
Alanine racemase, biosynthetic; Alanine racemase	alr	1.13	-0.22	-0.46
D-serine/D-alanine/glycine transporter	cycA	0.75	-0.58	0.66
Membrane-bound lytic murein transglycosylase A	mltA	0.86	-0.25	-0.32
Membrane-bound lytic murein transglycosylase B	mltB	1.61	0.21	-0.5
N-acetylmuramoyl-L-alanine amidase amiB	amiB	0.7	1.23	0.38
Penicillin-binding protein 1A	mrcA	1.11	0.19	0.13
Penicillin-binding protein 1B	mrcB	0.59	0.94	0.66
Penicillin-binding protein 1C	pbpC	0.9	0.09	0.18
Probable N-acetylmuramoyl-L-alanine amidase amiA	amiA	0.42	-1.49	-1.49
Protein hipA	hipA	0.73	-0.54	-0.18
Rod shape-determining protein mreC	mreC	1.38	-0.26	0.49
UDP-N-acetylenolpyruvoylglucosamine reductase	murB	0.66	-0.32	-1.43
phosphotransferase system EIIB				
Ascorbate-specific permease IIC component ulaA	ulaA	0.91	-0.4	
Glucitol/sorbitol permease IIC component	srlA	1.36	-0.16	0.58
N-acetylgalactosamine permease IID component	agaD	1.52	0.7	0.89
enoyl coa hydratase activity; caprolactam degradation				
c5023	c5023	0.9	-0.57	NS
galactose metabolism				
6-phosphofructokinase; 6-phosphofructokinase isozyme 1	pfkA	0.63	1.48	0.23
Beta-galactosidase	lacZ	1.55	0.08	0.56
D-tagatose-1,6-bisphosphate aldolase subunit gatY	gatY	1.3	-0.31	-0.18

D-tagatose-1,6-bisphosphate aldolase subunit gatZ	gatZ	1.17	NS	0.18
Evolved beta-galactosidase subunit alpha	ebgA	1.11	0.34	0.18
Galactitol-1-phosphate 5-dehydrogenase	gatD	1.77	-0.14	-0.38
Maltodextrin glucosidase	malZ	1.83	0.16	
L-phen-metabolic process				
Aromatic amino acid transport protein aroP	aroP	0.55	-0.4	0.32
Phe operon leader peptide	pheL	0.26	0.17	-0.1
Phenylalanine-specific permease	pheP	0.78	-0.47	-0.16
Phospho-2-dehydro-3-deoxyheptonate aldolase, Phe-sensitive	aroG	0.36	-0.87	-1.23
Glycosyl transferase/Peptidoglycan biosynthesis				
ECIAI1_3796; ECIAI39_4144; EcSMS35_3961; EcHS_A3835	waaW	0.51	3.37	1.25
Lipopolysaccharide 1,2-glucosyltransferase	rfaJ	0.71	2.94	2.6
Lipopolysaccharide 1,3-galactosyltransferase	rfaI	0.91	2.84	1.76
aromatic amino acid family biosynthetic process				
3-phosphoshikimate 1-carboxyvinyltransferase	aroA	0.96	0.58	0.13
Anthranilate synthase component 1	trpE	1.05	-0.9	-1.19
Chorismate synthase	aroC	0.86	-0.9	-1.05
Low affinity tryptophan permease	tnaB	0.83	0.4	0.28
Tryptophan-specific transport protein	mtr	1.27	-0.39	-1.39
c1220	c1220	1.2	0.29	0.26
DNA helicase-UVr				
ATP-dependent DNA helicase rep	rep	1.77	-0.32	0.75
Exodeoxyribonuclease V beta chain	recB	1.1	0.2	-0.14
ATPase activity, coupled to movement of substances				
Alpha-hemolysin translocation ATP-binding protein hlyB	hlyB	1.09	-0.7	-0.57
Lead, cadmium, zinc and mercury-transporting ATPase	zntA	1.2	-0.48	-0.16
Magnesium-transporting ATPase, P-type 1	mgtA	1.2	0.35	0.16
Multidrug resistance-like ATP-binding protein mdlA	mdlA	0.94	-0.65	0.82

UTI89_C4007; Z4885; c4286	yhiH	1.13	0.52	0.12
c0361	c0361	1.22	0.43	NS
c1232	mchF	0.9	-0.23	NS
c1820	c1820	0.74	-0.71	-1.24
c3292	c3292	1.07	2.83	2.09
Selenoamino acid metabolism				
Cystathionine gamma-synthase	metB	0.81	-0.33	-0.38
Cysteine synthase B; Cysteine synthase	cysM	1.14	-1.48	-1.02
Methionyl-tRNA synthetase	metG	1.05	-0.33	NS
Arginine-proline metabolism				
Acetylornithine deacetylase	argE	1.48	-0.28	-1.19
Argininosuccinate lyase	argH	0.9	0.49	1
Bifunctional protein putA	putA	1.2	-0.21	NS
Cytosine deaminase; Protein ccdA	codA	1.2	-0.27	-0.16
Glutamate 5-kinase	proB	0.89	-0.45	-1.1
N-succinylglutamate 5-semialdehyde dehydrogenase	astD	0.73	0.63	1.02
Ornithine carbamoyltransferase chain I	argI	0.6	-0.65	-0.16
Arginine N-succinyltransferase	astA	1.48	NS	0.18
N-succinylarginine dihydrolase	c2145	1.77	NS	0.59
Pentose and glucuronate interconversions				
Altronate hydrolase	uxaA	0.59	1.94	0.56
ECIAI1_3749; EC55989_4039; ECDH10B_3763; c4405; UTI89_C4126;	sgbU	1.2	0.15	NS
EcHS_A3787; c4406; ECDH10B_3764; ECUMN_4096; UTI89_C4127	sgbE	1.11	0.19	0.66
L-arabinose isomerase	araA	0.9	0.55	-0.55
L-xylulose/3-keto-L-gulonate kinase	lyxK	1.35	-0.58	-0.32
Rhamnulokinase	rhaB	1.1	0.09	0.56
Rhamnulose-1-phosphate aldolase	rhaD	1.45	-0.16	-0.13

Xylulose kinase	xylB	1.89	0.53	0.38
Histidine metabolism				
Histidine biosynthesis bifunctional protein hisB	hisB	1.12	0.42	-0.28
Histidine biosynthesis bifunctional protein hisIE	hisI	1.21	1.06	0.18
Histidinol dehydrogenase	hisD	1.06	1.06	0.88
Fructose-mannose-metabol				
L-rhamnonate dehydratase	rhmD	0.86	-0.41	0.15
Mannitol-1-phosphate 5-dehydrogenase	mtlD	1.27	1.01	1.45
Mannose-6-phosphate isomerase	manA	0.4	-1.51	-2.2
Biotin metabolism				
Bifunctional protein birA	birA	0.54	0.39	NS
Biotin synthase	bioB	1.36	-2.04	-2.43
Putative dethiobiotin synthetase; Dethiobiotin synthetase	bioD	1.67	NS	-0.07
Fatty acid oxidation				
Propionate catabolism operon regulatory protein	prpR	1.27	0.29	
starch and sucrose metabolism				
Alpha-amylase	malS	0.61	-0.22	0.13
Periplasmic beta-glucosidase	bglX	1.14	NS	0.75
Phosphorylase; Glycogen phosphorylase	glgP	1.05	2.16	1.66
Phosphorylase; Maltodextrin phosphorylase	malP	1.05	1.15	1.17
Putative beta-phosphoglucomutase	ycjU	1.19	0.31	-0.14
Putative sucrose phosphorylase	ycjM	2.02	-0.36	-0.25
Phantothenate and CoA				
3-methyl-2-oxobutanoate hydroxymethyltransferase	panB	0.87	-0.15	NS
D-phenylhydantoinase	ygeZ	1.92	-0.6	0.43
Pantothenate synthetase	panC	0.85	-0.61	-0.4
Sodium/pantothenate symporter	panF	1.11	NS	0.26
Taurine and hypotaurine metabol				

Alpha-ketoglutarate-dependent taurine dioxygenase	tauD	1.54	0.79	0.65
Amino sugar-nucleotide-sugar-me				
Probable bifunctional chitinase/lysozyme	chiA	0.83	-0.53	-0.6
Bifunctional polymyxin resistance protein arnA	yfbG	1.13	2.83	-0.19
N-acetylmuramic acid 6-phosphate etherase	murQ	1.09	-0.37	-0.33
Adenylate cyclase activity				
Adenylate cyclase	cyaA	1.09	0.66	-0.5
Formate hydrogenlyase transcriptional activator	fhlA	0.61	2.53	1.28
c3570; pO157p18; p026VIR_p015; UTI89_C4926	hlyA	0.36	0.19	-0.38
PyruvateFL-act-Enzyme				
Uncharacterized protein yjjW	yjjW	0.77	NS	1.42
c0909	c0909	1.34	0.2	0.19
c4538	c4538	1.17	0.39	0.18
pyruvate metabolism				
Aldehyde dehydrogenase B	aldB	0.65	-0.76	1.56
L-lactate dehydrogenase [cytochrome]	lldD	0.78	-0.37	0.68
Malate synthase A; Malate synthase	aceB	0.89	-1.77	-2
Pyruvate dehydrogenase [cytochrome]	poxB	0.58	2.03	0.95
Glyoxylate carboligase	gcl	1.05	NS	-0.4
TCA cycle				
Aconitate hydratase 2	acnB	0.59	0.12	0.4
Fumarate hydratase class I, anaerobic	fumB	0.91	2.02	0.85
Malate synthase G	glcB	1.35	NS	0.25
Succinate dehydrogenase flavoprotein subunit	sdhA	0.83	-0.29	0.82
Succinyl-CoA ligase [ADP-forming] subunit alpha	c5037	0.72	0.29	0.2
Nitrogen metabolism				
Nitrite reductase [NAD(P)H] large subunit	nirB	1.3	4.07	0.41
Respiratory nitrate reductase 2 alpha chain	narZ	0.95	0.76	0.11

Respiratory nitrate reductase 2 beta chain	narY	1.09	0.32	-0.2
Respiratory nitrate reductase 2 delta chain	narW	0.75	-0.4	0.55
Respiratory nitrate reductase 2 gamma chain	narV	1.11	0.54	1.34
Cysteine-methionine metabolism				
5-methyltetrahydropteroyltriglutamate	metE	1.07	-0.66	-0.08
Methionine synthase	metH	1.22	0.32	0.43
Pyrimidine metabolism				
AMP nucleosidase	amn	0.93	-0.28	-0.29
Anaerobic ribonucleoside-triphosphate reductase	nrdD	1.13	0.29	1.16
GMP synthase [glutamine-hydrolyzing]	guaA	1.14	0.12	-0.32
Inosine-5'-monophosphate dehydrogenase	guaB	0.57	-1.54	-3.58
Phosphoribosylamine--glycine ligase	purD	1.12	2.23	NS
Phosphoribosylformylglycinamidine synthase	purL	0.88	0.88	0.89
Purine nucleoside phosphorylase deoD-type	deoD	0.91	1.34	0.97
Pyrimidine-specific ribonucleoside hydrolase rihB	rihB	0.95	0.3	0.14
Ribonucleoside-diphosphate reductase 1 subunit beta	nrdB	0.46	0.6	-0.45
Ribonucleoside-diphosphate reductase 2 subunit beta	nrdF	1.35	-0.23	-0.27
Xanthosine phosphorylase	xapA	1.07	-0.57	NS
Thymidine phosphorylase	deoA	1.21	0.64	0.95
Carbamoyl-phosphate synthase large chain	carB	1.13	0.95	0.43
Mannitol dehydrogenase				
c1968	ydfI	1.45	0.07	0.39
c3751	c3751	0.69	-0.32	NS
Ascobate-aldarate metabolism				
L-ribulose-5-phosphate 4-epimerase ulaF	sgaE	1.55	0.77	NS
Ketose-bisphosphate aldolase, class-II				
c4483	c4483	1.54	-0.32	-0.19
c4484	c4484	0.96	-0.36	0.18

Sulfur metabolism				
Phosphoadenosine phosphosulfate reductase	cysH	0.73	0.51	0.4
Sulfite reductase [NADPH] hemoprotein beta-component	cysI	0.86	0.08	0.55
replication fork				
ATP-dependent DNA helicase recQ	recQ	1.13	-1.8	-1.88
Primosomal protein N'	priA	0.85	-0.27	-0.75
Replicative DNA helicase	dnaB	0.56	-1.15	-1.64
Ornitinr-lysine-arginine-decarboxylase				
c4501	c4501	1.23	-0.91	NS
Vit B6 metabol-process				
1-deoxy-D-xylulose-5-phosphate synthase	dxs	1.12	NS	-0.36
4-hydroxythreonine-4-phosphate dehydrogenase	pdxA	1.13	-0.72	-0.32
Erythronate-4-phosphate dehydrogenase	pdxB	0.36	-0.56	-1.54
fibronectin type III				
c1466	c1466	1.31	0.31	0.4
c1590	c1590	1.23	0.53	0.32
c3154	c3154	0.68	-0.17	
Peptidase M24 meth-aminopeptida				
Xaa-Pro aminopeptidase	pepP	1.11	0.29	0.47
c2924	c2924	1.61	0.31	-0.12
Glycerophospholipid metabolism				
Aerobic glycerol-3-phosphate dehydrogenase	glpD	0.81	-3.01	-0.73
Anaerobic glycerol-3-phosphate dehydrogenase subunit A	glpA	0.94	0.25	1.27
Anaerobic glycerol-3-phosphate dehydrogenase subunit B	glpB	1.13	0.71	1.71
Anaerobic glycerol-3-phosphate dehydrogenase subunit C	glpC	1.36	-0.31	
CDP-diacylglycerol pyrophosphatase	cdh	0.76	0.34	-0.24
Phospholipase A1	pldA	0.79	-0.78	-1.39

Haloacid dehydrogenase/epoxide hydrolase				
Phosphatase yieH	yieH	1.11	NS	0.36
Phosphatase yihX	yihX	0.78	0.43	-0.64
Phosphopantetheine binding				
c2459	c2459	1.11	0.19	0.2
c2468	c2468	0.4	3.15	1.61
c3714	c3714	0.75	0.49	-1.19
Glycerolipid metabolism				
Glycerol kinase	glpK	0.69	0.14	1.34
Phosphoglycerol transferase I	mdoB	0.89	-0.98	-0.46
thiamin and derivative metabolic process				
Hydroxyethylthiazole kinase	thiM	1.14	-0.44	-0.31
Thiamine biosynthesis protein thiC	thiC	0.91	1.42	0.68
Glycolysis / Gluconeogenesis				
6-phospho-beta-glucosidase bglA	bglA	0.72	0.43	NS
Ethanolamine utilization protein eutG	eutG	2.22	0.53	NS
Glucose-1-phosphatase	agp	0.7	0.87	2.13
Phosphoglucomutase	pgm	0.92	0.71	-0.2
RNA methylation				
Ribosomal RNA large subunit methyltransferase G	ygjO	1.13	-0.66	-0.26
Ribosomal RNA large subunit methyltransferase L	rlmL	0.69	-0.22	0.39
Ribosomal RNA small subunit methyltransferase B	sun	0.76	NS	-0.16
Ubiquinone-terpenoid				
2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase	yfbB	1.78	0.39	NS
2-succinylbenzoate--CoA ligase	menE	1.22	-0.44	-0.31
Chorismate--pyruvate lyase	ubiC	0.72	-0.43	-1.17
o-succinylbenzoate synthase	menC	0.84	1.56	0.7
Serine-type endopeptidase				

Protease 2	ptrB	0.76	-0.1	-0.64
Protease degS	degS	1.23	-0.46	NS
Serine protease pic	c0350	0.82	0.43	0.25
Serine protease sat; Streptothricin acetyltransferase	sat	1.12	-0.2	-0.26
c0393	c0393	1.07	-0.18	-0.32
c3190	c3190	1.14	-0.27	0.15
TPR repeat				
Cytochrome c-type biogenesis protein ccmH	ccmH	0.95	1.28	1.25
Protein hemY	hemY	0.91	2.13	0.65
TPR repeat-containing protein yfgC	yfgC	1.26	0.61	0.32
Regulation cell morphogenesis				
Cytoskeleton protein rodZ	yfgA	0.47	-1.95	-2.42
Rod shape-determining protein rodA	mrdB	0.72	0.19	-0.32
Cytolysis				
Lysis protein S homolog from lambdoid prophage DLP12	c1561	0.34	-0.58	-0.43
Lysozyme	c3180	1.22	-0.4	0.16
Lysozyme	c0957	1.59	NS	-0.19
Lysozyme	c1436	1.11	0.58	0.14
Microcin H47	mchB	1.87	-0.57	0.25
c1437	c1437	1.23	NS	0.16
RNA degradation				
Poly(A) polymerase	pcnB	0.63	-2.21	-2.03
Ribonuclease E	rne	0.6	NS	-1.07
Helix-turn-helix, AraC type				
HTH-type transcriptional regulator gadW	yhiW	0.68	2.46	0.15
HTH-type transcriptional regulator gadX	yhiX	0.49	0.24	-1.61
Regulatory protein ada	ada	1.94	0.73	0.2
Uncharacterized HTH-type transcriptional regulator ykgD	ykgD	0.47	-0.54	-0.84

Xylose operon regulatory protein	xylR	1.16	-0.16	0.18
c1810	c1810	0.84	-0.92	-1.39
c2971; ECS88_2625; ECED1_2879; ECDH10B_2602	yfeG	0.75	-0.13	0.19
c4546	c4546	1.2	-0.23	NS
c4549	c4549	1.19	-0.43	-0.24
Tetrapyrrole metabolic process				
Glutamyl-tRNA reductase	hemA	0.48	-3	-3.27
Oxygen-independent coproporphyrinogen-III oxidase-like protein	yggW	1.13	-0.27	NS
Uncharacterized protein yifB	yifB	1.54	0.32	0.18
c4314; E2348C_3745; UTI89_C4034; APECO1_2946; Z4914	chuW	1.37	-0.49	0.31
Solute:hydrogen antiporter activity				
Glutathione-regulated potassium-efflux system protein kefB	kefB	1.16	1.01	0.5
Putative multidrug resistance protein mdtD	yegB	0.84	-0.23	-0.17
c5057	c5057	0.75	0.74	0.96
c5435	c5435	1.38	0.15	-0.61
Lipopolysaccharide biosynthesis				
Lipopolysaccharide core biosynthesis protein rfaY	rfaY	0.34	3.26	2.44
Lipopolysaccharide heptosyltransferase 1	rfaC	1.1	0.23	-0.26
DNA-methylation				
c0941	c0941	0.88	0.07	-0.2
c5373	c5373	1.23	-0.23	0.14
p1ECUMN_0135; UTI89_P077; O2R_81; c2876; LH0092; Z3593	yfcB	1.17	-1.03	-1.34
Aminoacyl-tRNA ligase activity				
Glutamyl-Q tRNA(Asp) synthetase; Insertion element IS150	yadB	0.62	0.89	-1.43
Prolyl-tRNA synthetase	proS	1.1	-1.46	-1.19
tRNA(Ile)-lysidine synthase	tilS	0.88	-0.25	NS
TonB-dependent receptor, plug				
ECUMN_4000; UTI89_C4028; Z4911; ECIAI39_4002	chuA	0.96	-1.01	NS

c1265	c1265	0.5	-1.11	-1.19
c2518	c2518	0.34	1.34	0.64
c3775	c3775	0.91	-0.76	-0.82
Amino acid/polyamine transporter I				
c4500	c4500	0.94	0.15	0.38
c4502	c4502	1.57		0.43
Glyoxylate and dicarboxylate metabolism				
Formate dehydrogenase-O major subunit	fdoG	0.78	0.32	0.45
rRNA metabolic process				
23S rRNA (uracil-5-)-methyltransferase rumB	rumB	0.95	-0.61	-0.32
Ribosomal large subunit pseudouridine synthase F	yjbC	0.81	-0.27	-0.35
Ribosomal large subunit pseudouridine synthase E	ymfC	0.36	-1.84	-2.52
Protein secretion				
APECO1_1740; Z0290; E2348C_0224; ECIAI1_0270; EcE24377A_0263	fhiA	0.86	0.25	-0.48
APECO1_490; UTI89_C4924; pO157p20; c3574; p026VIR_p017	hlyD	0.87	-0.31	0.95
Multidrug resistance protein mdtN	yjcR	1.35	NS	-0.07
Protein transport protein hofC	hofC	1.38	0.4	NS
UTI89_C3780; c4096	yheF	0.78	-1.57	-0.79
c0362	c0362	1.97	-0.27	NS
c1231	mchE	1.1	-0.66	-0.18
c1811	c1811	1.85	-0.29	NS
c3702	c3702	1.17	0.32	NS
c4097; UTI89_C3781	yheG	0.91	-0.4	-0.55
c4103; UTI89_C3787	yheJ	1.2	NS	0.14
Nicotinamide nucleotide metabolic process				
Soluble pyridine nucleotide transhydrogenase	udhA	0.7	NS	0.19
Transcriptional regulator nadR	nadR	0.81	0.93	0.73
Transketolase 1	tktA	0.88	NS	-0.3

Transketolase 2	tktB	0.63	2.4	1.48
Uncharacterized oxidoreductase yihU	yihU	0.86	-0.77	0.15
Molybdopterin cofactor biosynthetic process				
Molybdopterin biosynthesis protein moeA	moeA	0.7	-1.05	-2
Organelle envelope				
2,3-diketo-L-gulonate TRAP transporter large permease protein yiaN	yiaN	1.35	0.11	-0.07
Acriflavine resistance protein F	acrF	1.2	-0.29	-0.92
Apolipoprotein N-acyltransferase	lnt	1.12	1.12	-0.18
Cytochrome c-type biogenesis protein nrfE	nrfE	1.49	0.32	NS
DNA translocase ftsK	ftsK	0.95	-0.19	0.28
Ethanolamine utilization protein eutH	eutH	0.65	-0.18	NS
Formate hydrogenlyase subunit 3	hycC	1.37	1.09	0.36
Hexuronate transporter	exuT	0.52	0.79	1.47
High-affinity gluconate transporter	gntP	1.54	0.22	0.09
Inner membrane ABC transporter permease protein ynjC	ynjC	0.9	0.25	0.2
Inner membrane metabolite transport protein ygcS	ygcS	1.1	-0.1	NS
Inner membrane protein yhjX	yhjX	0.9	-1.14	-0.99
Inner membrane protein yqiK	yqiK	0.67	-0.2	0.25
Inner membrane transport protein ydiM	ydiM	1.73	-0.44	-0.46
Multidrug resistance protein B	emrB	1.17	-0.62	0.58
Multidrug transporter emrE	emrE	0.8	-0.32	-0.59
NADH-quinone oxidoreductase subunit M	nuoM	0.63	1.16	0.83
Na(+)/H(+) antiporter nhaA	nhaA	0.93	0.43	0.7
Nucleoside permease nupC	nupC	0.63	-0.77	-0.25
Probable low-affinity inorganic phosphate transporter 2	pitB	2.16	-0.54	NS
Protein abrB	abrB	1.26	1.69	0.41
Protein fdrA	fdrA	1.11	NS	0.19
Protein phnM	phnM	1.22	0.2	-0.24

Putative amino-acid ABC transporter permease protein yhdX	yhdX	0.67	0.23	-0.15
Putative metabolite transport protein ydjK	ydjK	0.81	0.87	1.03
Quinoprotein glucose dehydrogenase	gcd	1.14	-1.1	-0.4
Sodium/glutamate symport carrier protein	gltS	0.93	0.5	0.59
Threonine/serine transporter tdcC	tdcC	1.05	-0.43	0.88
Uncharacterized symporter yidK	yidK	1.33	-0.15	0.19
Xanthosine permease	xapB	0.68	-0.32	-0.22
Acyl-CoA N-acyltransferase				
EcHS_A4007; EcSMS35_4154; Z5301; ECH74115_5223; c4710	wecD	1.58	0.74	0.25
Protein elaA	elaA	1.11	-0.57	-0.4
Protein phnO	phnO	0.63	-0.53	-1.36
c5355	c5355	0.82	0.43	-0.6
Regulation of cellular protein metabolic process				
N-acetylglucosamine repressor	nagC	0.66	-0.44	-0.78
Selenocysteine-specific elongation factor	selB	0.82	0.27	-0.15
Uncharacterized protein yciH	yciH	1.29	-1.65	-0.25
Palmitate				
Endo-type membrane-bound lytic murein transglycosylase A	mltE	1.2	-0.09	-0.25
Outer-membrane lipoprotein lolB	lolB	0.91	-0.46	-0.66
Small protein A	bamE	0.7	-0.72	-1.53
Uncharacterized lipoprotein ybjP	ybjP	0.91	0.26	-0.15
Uncharacterized lipoprotein yfhM	yfhM	0.82	0.77	0.38
Uncharacterized lipoprotein ypdI	ypdI	1.35	-0.4	NS
Uncharacterized protein ytcA	c5088	1.33	0.19	0.13

NS= not significant